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(54) Title: NOVEL POLYKETIDE DERIVATIVES AND RECOMBINANT METHODS FOR MAKING SAME

(57) Abstract

The invention provides novel erythromycin derivatives in which methyl groups on the macrolactone ring have been substituted with –H, –Et, and/or –OH and the ethyl side–chain has been substituted with hydroxymethyl or dihydroxyxyclohexylmethyl side–chains. The invention also provides reagents such as isolated polynucleotides, vectors comprising the polynucleotides and host cells transformed with the vectors for making the novel compounds. Methods for making the compounds utilizing genetic engineering techniques are also disclosed.

NOVEL POLYKETIDE DERIVATIVES AND RECOMBINANT
METHODS FOR MAKING SAME

This application is a continuation-in-part of co-pending U.S. Serial No. 08/858,003,
filed May 16, 1997 which is a continuation-in-part of co-pending U.S. Serial No. 07/642,734,
filed January 17, 1991.

Technical Field

The present invention relates to novel polynucleotide sequences, proteins encoded
therefrom which are involved in the biosynthesis of polyketides, methods for directing the
biosynthesis of novel polyketides using those polynucleotide sequences and novel derivatives
produced therefrom. In particular, the invention relates to the production of novel polyketide
derivatives through manipulation of the genes encoding polyketide synthases.

Background of the Invention

Polyketides are a large class of natural products that includes many important
antibiotic, antifungal, anticancer, antihelminthic, and immunosuppressant compounds such as
erythromycins, tetracyclines, amphotericins, daunorubicins, avermectins, and rapamycins.
Their synthesis proceeds by an ordered condensation of acyl esters to generate carbon chains
of varying length and substitution pattern that are later converted to mature polyketides. This
process has long been recognized as resembling fatty acid biosynthesis, but with important
differences. Unlike a fatty acid synthase, a typical polyketide synthase is programmed to
make many choices during carbon chain assembly: for example, the choice of "starter" and
"extender" units, which are often selected from acetate, propionate or butyrate residues in a
defined sequence by the polyketide synthase. The choice of using a full cycle of reduction-
dehydration-reduction after some condensation steps, omitting it completely, or using one of
two incomplete cycles (reduction alone or reduction followed by dehydration) is additionally
programmed, and determines the pattern of keto or hydroxyl groups and the degree of
saturation at different points in the chain. Finally, the stereochemistry for the substituents at
many of the carbon atoms is programmed by the polyketide synthase.

Streptomyces and the closely related Saccharopolyspora genera are producers of a
prodigious diversity of polyketide metabolites. Because of the commercial significance of
these compounds, a great amount of effort has been expended in the study of Streptomyces
and Saccharopolyspora genetics. Consequently, much is known about these organisms and
several cloning vectors and techniques exist for their transformation.

Although many polyketides have been identified, there remains the need to obtain
novel polyketide structures with enhanced properties. Current methods of obtaining such

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molecules include screening of natural isolates and chemical modification of existing

polyketides, both of which are costly and time consuming. Current screening methods are

based on gross properties of the molecules, i.e. antibacterial, antifungal activity, etc., and both

a priori knowledge of the structure of the molecules obtained or predetermination of

5   enhanced properties are virtually impossible. Chemical modification of preexisting structures

has been successfully employed to obtain novel polyketides, but still suffers from practical

limitations to the type of compounds obtainable, largely connected to the poor yield of

multistep synthesis and available chemistry to effect modifications. Modifications which are

particularly difficult to achieve are those involving additions or deletions of carbon side

10   chains. Accordingly, there exists a considerable need to obtain molecules wherein such

changes can be specified and performed in a cost effective manner and with high yield.

The present invention solves these problems by providing reagents (specifically,

polynucleotides, vectors comprising the polynucleotides and host cells comprising the

vectors) and methods to generate novel polyketides by *de novo* biosynthesis rather than by

15   chemical modification.

Summary of the Invention

In one aspect, the present invention provides compounds of the formula:
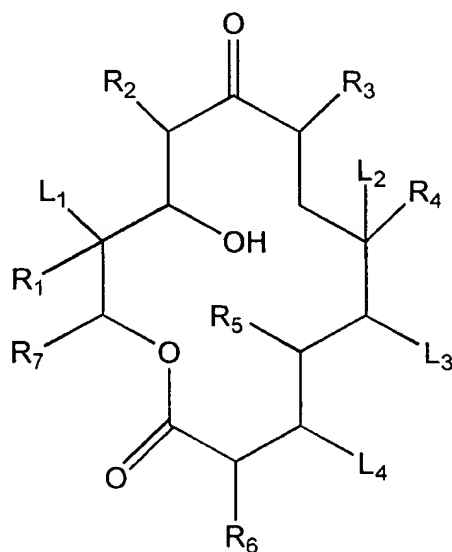
X

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wherein R_1, R_2, R_3, R_4, R_5, and R_6 are independently selected from Q wherein Q is selected
from the group consisting of (a) -H, (b) -Me, (c) -Et, and (d) -OH; R_7 is selected from the
group consisting of -Et, -HOMe, and 13-3,4-dihydrocyclohexylmethyl; L_1 and L_2 are

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independently -H or -OH; L₃ is D-desosamine or -OH; and L₄ is L-mycarose, L-cladinose or -OH with the proviso that when R_7 is -Et and R_1-R_5 are -Me, R_6 is other than -H or -Me.

Preferred compounds of the invention are those in Q is selected from the group consisting of (a), (b) and (c) above or (a), (b) and (d) above or (a), (c) and (d) above or (b), (c) and (d)

5   above or (a) and (b) above or (a) and (c) above or (a) and (d) above or (b) and (c) above or (c) and (d) above, R_7 is -Et and L_1, L_2, L_3, and L_4 are as defined above. Other preferred compounds include those in which R_1, R_2, R_3, R_4, R_5, and R_6 are all -H or -Et or -OH, R_7 is -Et and L_1, L_2, L_3 and L_4 are as defined above. Still other preferred compounds include didesmethyl, tridesmethyl, tetradesmethyl, pentadesmethyl, and hexadesmethyl derivatives of

10   the compounds of formula X and particularly, di- tri-, tetra-, penta-, and hexadesmethyl derivatives of erythromycins A and B. Other especially preferred compounds of formula X include 6,10-didesmethyl-6-ethylerythromycin A, 10,12-didesmethyl-12-deoxy-12-ethylerythromycin A, 10,12-didesmethyl-12-deoxy-10-hydroxyerythromycin A, 6,10,12-tridesmethyl-6,12-diethylerythromycin A, 6,10,12-tridesmethyl-6-deoxy-6,12-

15   diethylerythromycin A, 10-desmethylerythronolide B, 10-desmethyl-6-deoxyerythronolide B, 12-desmethylerythronolide B, 12-desmethyl-6-deoxyerythronolide B, 12-desmethyl-12-ethylerythronolide B, 6-desmethyl-6-deoxy-6-ethylerythronolide B, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 10-desmethyl-6,12-dideoxyerythromycin A, 12-desmethylerythromycin A, 12-desmethyl-12-deoxyerythromycin

20   A, 12-desmethyl-6,12-dideoxyerythromycin A, 6-desmethyl-6-ethylerythromycin A, 12-desmethyl-12-ethylerythromycin A, 12-desmethyl-12-deoxy-12-ethylerythromycin A, 10-desmethyl-10-hydroxyerythromycin A, 12-desmethyl-12-epihydroxyerythromycin A, 10,12-didesmethylerythromycin A, 10,12-didesmethyl-12-deoxyerythromycin A, 10,12-didesmethyl-6,12-dideoxyerythromycin A, 10-desmethylerythronolide B, 10-desmethyl-6-

25   deoxyerythronolide B, 12-desmethylerythronolide B, 12-desmethyl-6-deoxyerythronolide B, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 10-desmethyl-6,12-dideoxyerythromycin A, 12-desmethylerythromycin A, 12-desmethyl-12-deoxyerythromycin A, 12-desmethyl-6,12-dideoxyerythromycin A, 10,12-didesmethylerythromycin A, 10,12-didesmethyl-12-deoxyerythromycin A, and 10,12-didesmethyl-6,12-dideoxyerythromycin A.

30   Most preferred compounds include 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 12-desmethyl-12-deoxyerythromycin A, 8-desmethyl-8-hydroxyerythromycin A, 6-desmethyl-6-epierythromycin A, 4-desmethyl-4-hydroxyerythromycin A, 2-desmethyl-2-hydroxyerythromycin A, 13-desethyl-13-hydroxymethol erythromycin A, 2,12-didesmethyl-2,12-dihydroxyerythromycin, 4,10-

35   didesmethyl-4,10-dihydroxyerythromycin, 10,12-didesmethyl-10-hydroxyerythromycin,

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6,10-didesmethyl-6-ethyl-10-hydroxyerythromycin A, and 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A.

In another aspect, the present invention provides an isolated polynucleotide sequence or fragment thereof which encodes an enzymatically active acyltransferase domain from a PKS selected from *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, and *Streptomyces caelestis*. Preferably, the polynucleotide sequence is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:29 or SEQ ID NO:30. In another preferred embodiment, the polynucleotide sequence encodes an acyltransferase domain selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33 and SEQ ID NO:34.

The present invention also provides a vector comprising a polynucleotide sequence or fragment thereof which encodes which encodes an enzymatically active acyltransferase domain from *Streptomyces*. Preferably, the polynucleotide sequence is selected from those described above and the *Streptomyces* is *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, or *Streptomyces caelestis*. A particularly preferred vector is pCS5. Other vectors of the invention include pUC18/LigAT2, pEryAT1/LigAT2, pEryAT2/LigAT2, pUC18/venAT, pEryAT1/venAT, pUC19/rapAT14, pEryAT1/rapAT14, pEryAT2/rapAT14, pUC/5'-flank/ethAT, pUC/ethAT/C-6, pEAT4, pUC18/NidAT6, pEryAT2/NidAT6. pEryATs/NidAT6 and pEryATs/rapligase 3.0.

In another aspect, the invention provides host cells transformed with a vector as described above. The host cell may be a bacterial cell and preferably is selected from the group consisting of *E. coli* and *Bacillus* species. Alternatively, the host cell is a polyketide-producing microorganism. A preferred polyketide-producing host cell is selected from the group consisting of *Saccharopolyspora* species, *Nocardia* species, *Micromonospora* species, *Arthrobacter* species, *Streptomyces* species, *Actinomadura* species, and*Dactylosporangium*. species. An even more preferred polyketide-producing host cell is selected from the group consisting of *Saccharopolyspora hirsuta*, *Micromonospora rosaria*, *Micromonospora megalomicea*, *Streptomyces antibioticus*, *Streptomyces mycarofaciens*, *Streptomyces avermitilis*, *Streptomyces hygroscopicus*, *Streptomyces caelestis*, *Streptomyces tsukubaensis*, *Streptomyces fradiae*, *Streptomyces platensis*, *Streptomyces violaceoniger*, *Streptomyces ambofaciens*, *Streptomyces griseoplanus*, and *Streptomyces venezuelae*. Of these host cells, *Saccharopolyspora erythraea*, *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, and *Streptomyces caelestis* are most preferred.

The invention also provides a method for altering the substrate specificity of a polyketide synthase in a first polyketide-producing microorganism comprising the steps of (a) isolating a first and second genomic DNA segment, each comprising a polyketide synthase wherein the first genomic DNA segment is from the first polyketide-producing

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microorganism and the second genomic DNA segment is from the first polyketide-producing microorganism or a second polyketide-producing microorganism;

(b)   identifying one or more discrete fragments of the first genomic DNA segment, each of which encodes an acyltransferase domain;

(c)   identifying one or more discrete fragments of the second genomic DNA segment, each of which encodes a related domain to the acyltransferase domain of the first genomic DNA segment; and

(d)   transforming a cell of the first polyketide-producing microorganism with one or more of the fragments from step (c) under conditions suitable for the occurrence of a homologous recombination event, leading to the replacement of one or more of the fragments from the first genomic DNA segment with one or more of the fragments from step (c). In one embodiment, the first polyketide-producing microorganism is *Saccharopolyspora erythraea* and the second polyketide-producing microorganism is *Streptomyces*. Preferred *Streptomyces* are selected from the group consisting of *Streptomyces antibioticus*, *Streptomyces mycarofaciens*, *Streptomyces avermitilis*, *Streptomyces hygroscopicus*, *Streptomyces caelestis*, *Streptomyces tsukubaensis*, *Streptomyces fradiae*, *Streptomyces platensis*, *Streptomyces violaceoniger*, *Streptomyces ambofaciens*, and *Streptomyces venezuelae*. Even more preferred *Streptomyces* are *Streptomyces caelestis*, *Streptomyces hygroscopicus*, or *Streptomyces venezuelae*. In a second embodiment, the first polyketide-producing microorganism is a *Streptomyces* as described above and the second polyketide-producing microorganism is *Saccharopolyspora erythraea*. Also in a preferred embodiment, the related domain is selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, and SEQ ID NO:34.

Brief Description of the Drawings

The present invention will be more readily appreciated in connection with the accompanying drawings.

FIG. 1 is a proposed metabolic pathway for the biosynthesis of erythromycin A in *Sac. erythraea*.

FIG. 2 is a schematic representation of the erythromycin PKS.

FIG. 3 is a Growtree analysis of AT domains from *Streptomyces hygroscopicus* (*S. hygroscopicus*; LigAT2 and rapAT1-14), *Streptomyces venezuelae* (*S. venezuelae*; venAT) and *Saccharopolyspora erythraea* (*Sac. erythraea*; eryAT1-6).

FIG. 4a is a schematic representation of gene replacements of EryAT1 with LigAT2 or venAT and EryAT2 with LigAT2 in *Sac. erythraea*.

FIG. 4b is a schematic representation of gene replacements of EryAT4 with an ethyl AT (NidAT5) in *Sac. erythraea*.

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FIG. 5 is a diagrammatic representation of gene replacement by homologous recombination.

FIG. 6 is a schematic representation of the genetic organization of the Ligase-PKS cluster from *S. hygroscopicus* ATCC 29253.

FIG. 7 represents the nucleotide sequence (SEQ ID NO:1, top strand) and corresponding amino acid sequence (SEQ ID NO:31, bottom strand) of LigAT, the malonyl AT domain from module 2 of the Ligase-PKS cluster of *S. hygroscopicus* ATCC 29253.

FIG. 8 is a diagrammatic representation of the strategy to clone the LigAT2 domain.

FIG. 9 is a flow diagram depicting the cloning of the EryAT1 flanking regions in plasmid pCS5.

FIG. 10 is a flow diagram depicting construction of pEryAT1/LigAT2.

FIG. 11 is a flow diagram depicting the cloning of the EryAT2 flanking regions in plasmid pCS5.

FIG. 12 is a flow diagram depicting construction of pEryAT2/LigAT2.

FIG. 13 represents the nucleotide sequence (SEQ ID NO:2, top strand) and corresponding amino acid sequence (SEQ ID NO:32, bottom strand) of venAT, the malonate AT domain from the PKS cluster (hereinafter designated pven4) from *S. venezuelae* ATCC 15439.

FIG. 14 is a diagrammatic representation of the strategy to clone the venAT domain.

FIG. 15 is a flow diagram depicting construction of pEryAT1/venAT.

FIG. 16 is a diagrammatic representation of the strategy to clone the rapAT14 domain.

FIG. 17 is a flow diagram depicting construction of pEryAT1/rapAT14.

FIG. 18 is a flow diagram depicting construction of pEryAT2/rapAT14.

FIG. 19 is a schematic representation of the genetic organization of the PKS cluster from *Streptomyces caelestis* NRRL-2821.

FIG. 20 is a diagram of the structure of the macrolide ring of niddamycin.

FIG. 21 represents the nucleotide sequence (SEQ ID NO:29, top strand) and corresponding amino acid sequence (SEQ ID NO:33, bottom strand) of NidAT5, the ethyl AT domain from module 5 of the PKS cluster of *Streptomyces caelestis* NRRL-2821.

FIG. 22 is a flow diagram depicting the construction of pUC/ethAT/C-6.

FIG. 23 is a diagram showing the nucleotide changes made to create an *Avr*II site at the 5' end of NidAT5.

FIG. 24 is a diagram of the replacement plasmid pEAT4.

FIG. 25 represents the nucleotide sequence (SEQ ID NO:30, top strand) and corresponding amino acid sequence (SEQ ID NO:34, bottom strand) of NidAT6, the AT domain in module 6 of the niddamycin PKS cluster.

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FIG. 26 is a diagrammatic representation of the strategy to clone the NidAT6 domain.

FIG. 27 is a flow diagram depicting construction of pEryAT2/NidAT6.

FIG. 28 is a flow diagram depicting the cloning of the EryATs flanking regions in plasmid pCS5.

FIG. 29 is a flow diagram depicting the construction of pEryATs/NidAT6.

FIG. 30 is a flow diagram depicting the cloning of pEryM1/NidAT6.

FIG. 31 is a flow diagram depicting the construction of the plasmid pSL1180/rapligase 3.0.

FIG. 32 is a flow diagram depicting construction of the plasmid pEryATs/rapligase 3.0.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions:

For the purposes of the present invention as disclosed and claimed herein, the following terms are defined:

The term "polyketide" as used herein refers to a large and diverse class of natural products including but not limited to antibiotic, anticancer, antihelminthic, antifungal, pigment, and immunosuppressant compounds. Antibiotics include but are not limited to anthracyclines, tetracyclines, polyethers, polyenes, ansamycins, and macrolides of various types such as avermectins, erythromycins, and niddamycins. The term polyketide is also intended to refer to compounds of this class that can be used as intermediates in chemical syntheses. For example, erythromycin A is a polyketide that is isolated and used in the synthesis of the antibiotic clarithromycin. Polyketides used as intermediates do not themselves necessarily have any biological or therapeutic activity.

The term "polyketide-producing microorganism" as used herein includes but is not limited to bacteria from the order *Actinomycetales*, *Myxococcales* or other *Eubacteriales* that can produce a polyketide. Examples of actinomycetes and myxobacteria that produce polyketides include but are not limited to *Saccharopolyspora erythraea*, *Saccharopolyspora hirsuta, Micromonospora rosaria, Micromonospora megalomicea, Sorangium cellulosum, Streptomyces antibioticus, Streptomyces mycarofaciens, Streptomyces avermitilis, Streptomyces hygroscopicus, Streptomyces caelestis, Streptomyces tsukubaensis, Streptomyces fradiae, Streptomyces platensis, Streptomyces violaceoniger, Streptomyces ambofaciens, Streptomyces venezuelae* and various other *Streptomyces, Actinomadura, Dactylosporangium* and *Amycolotopsis* strains that produce polyketides. Yeast and fungi that produce polyketides are also considered "polyketide-producing microorganisms". Examples

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of fungi that produce polyketides include but are not limited to members of the genus *Aspergillus*.

The term "polyketide synthase" (PKS) as used herein refers to a complex of enzyme activities responsible for the biosynthesis of polyketides. The enzymatic activities contained within a PKS include but are not limited to β-ketoreductase (KR), dehydratase (DH), enoylreductase (ER), β-ketoacyl ACP synthase (KS), acyl carrier protein (ACP), acyltransferase (AT) and thioesterase (TE). The polypeptide fragment responsible for each enzymatic activity is referred to as a "domain". A "module" refers to a group or set of domains which carry out one condensation step in the process of polyketide formation and may or may not include domains which effect processing of the β-carbonyl group in the growing polyketide.

The term "Type I PKS" as used herein refers to a PKS which is a large multifunctional protein and is exemplified by DEBS (see below). The term "Type II PKS" refers to a PKS having several separate, largely monofunctional enzymes, and is exemplified by the PKSs responsible for the biosynthesis of actinorhodin and tetracenomycin (C.R. Hutchinson and I. Fujii, *Annu. Rev. Microbiol.* 49:201-238 (1995)).

The term "cognate domains" as used herein refers to the members of a specific set of domains which constitute a naturally occurring single module.

The term "related domain" or "heterologous domain" as used herein refers to a PKS domain which is functionally similar to a second PKS domain. By "functionally similar" it is meant that each domain catalyzes a particular type of reaction but acts upon a different substrate. For example, the AT domain of module 1 of *Sac. erythraea* (eryAT1) and the AT domain of module 14 of *S. hygroscopicus* (rapAT14) both catalyze the transfer of an extender unit to a corresponding ACP domain. In the case of *Sac. erythraea*, however, eryAT1 utilizes methylmalonyl CoA as a substrate whereas in *S. hygroscopicus*, rapAT14 utilizes malonyl CoA. Thus, eryAT1 and rapAT14 are considered to be "related" or "heterologous" domains.

The term "condensation" as used herein refers to the addition of an extender unit to the nascent polyketide chain and requires the action of KS, AT and ACP domains of the PKS.

The term "starter" as used herein refers to a coenzyme A thioester of a carboxylic acid which is used by a polyketide synthase as the first building block of the polyketide.

The term "extender" as used herein refers to a coenzyme A thioester of a dicarboxylic acid that is incorporated into a polyketide by a polyketide synthase at positions other than the first position.

The term "DEBS" as used herein refers to the enzyme 6-deoxyerythronolide B synthase, the PKS that builds the polyketide-derived macrolactone 6-deoxyerythronolide B (6-DEB).

The term "*eryA*" as used herein refers to the genes which encode the DEBS.

The term "homologous recombination" as used herein refers to crossing over between DNA strands containing identical sequences.

The term "isolated" as used herein means that the material is removed from its original environment (e.g. the natural environment where the material is naturally occurring). For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, which is separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that the vector or composition is not part of the natural environment.

The term "restriction fragment" as used herein refers to any linear DNA generated by the action of one or more restriction enzymes.

The term "transformation" as used herein refers to the introduction of DNA into a recipient microorganism, irrespective of the method used for the insertion into the microorganism.

The term "replicon" as used herein means any genetic element, such as a plasmid, chromosome or virus, that behaves as an autonomous unit of polynucleotide replication within a cell. A "vector" is a replicon in which another polynucleotide fragment is attached, such as to bring about the replication and/or expression of the attached fragment.

The terms "recombinant polynucleotide" or "recombinant polypeptide" as used herein means at least a polynucleotide or polypeptide which by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide or polypeptide with which it is associated in nature and/or is linked to a polynucleotide or polypeptide other than that to which it is linked in nature.

The term "host cell" as used herein, refers to both prokaryotic and eukaryotic cells which are used as recipients of the recombinant polynucleotides and vectors provided herein.

The term "open reading frame" or "ORF" as used herein refers to a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

II. The Invention

In its broadest sense, the present invention entails novel polyketides with therapeutic activity (e.g. antimicrobial, anticancer, antifungal, immunosuppressant and/or antihelminthic

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activity) and immediate compounds of such polyketides. The invention also provides a
method for producing novel polyketides *in vivo* by selectively altering the genetic
information of an organism that naturally produces a polyketide. The present invention
further provides isolated and purified polynucleotides that encode PKS domains (i.e.

5   polypeptides) from polyketide-producing microorganisms, fragments thereof, vectors
containing those polynucleotides, and host cells transformed with those vectors. These
polynucleotides, fragments thereof, and vectors comprising the polynucleotides can be used
as reagents in the above described method. Portions of the polynucleotide sequences
disclosed herein are also useful as primers for the amplification of DNA or as probes to

10   identify related domains from other polyketide-producing microorganisms.

III. Polynucleotides

        The present invention provides isolated and purified polynucleotides that encode PKS
domains (i.e. polypeptides) and fragments thereof which are involved in the production of

15   polyketides. Polynucleotides included within the scope of the invention may be in the form
of RNA, DNA, cDNA, genomic DNA and synthetic DNA. The DNA may be double-
stranded or single-stranded, and if single-stranded may be the coding (sense) strand or non-
coding (anti-sense) strand. The coding sequence which encodes a polypeptide may be
identical to a coding sequence provided herein or may be a different coding sequence which,

20   as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide
as the DNA provided herein.

        Polynucleotides may include only the coding sequence for a particular polypeptide or
for a polypeptide which is functionally equivalent to the polypeptide sequences provided
herein. Additionally, the invention includes variant polynucleotides containing modifications

25   such as polynucleotide deletions, substitutions or additions; and any polypeptide modification
resulting from the variant polynucleotide sequence. A polynucleotide of the present
invention also may have a coding sequence which is a naturally occurring allelic variant of
the coding sequence provided herein.

        Probes and primers constructed according to the polynucleotide sequences provided

30   herein are also contemplated as within the scope of the present invention and can be used in
various methods to provide various types of analysis. For example, primer sequences may be
designed according to polynucleotide sequences which encode particular domains and then
used to amplify polynucleotide sequences of the same or other related domains using well-
known amplification techniques such as the polymerase chain reaction (PCR) and the ligase

35   chain reaction (LCR). (PCR has been disclosed in U.S. patents 4,683,195 and 4,683,202, and
LCR, in EP-A- 320 308 to K. Backman published June 16, 1989 and EP-A-439 182 to K.

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Backman *et al.*, published July 31, 1991, all of which are incorporated herein by reference).
Generation of primers for use in other amplification techniques or in variations of these
amplification techniques, (such as nested PCR) is also contemplated within the scope of the
invention and is considered within the knowledge of the routine practitioner.

5   Probes and primers may be designed from conserved nucleotide regions of a
polynucleotide of interest or from non-conserved nucleotide regions of a polynucleotide of
interest. Generally, nucleic acid probes are developed from non-conserved or unique regions
when maximum specificity is desired, and nucleic acid probes are developed from conserved
regions when assaying for nucleotide regions of related members of a multigene family or in
10   related species. Probes can also be labeled with radioisotopes or other detection labels for
screening of recombinant libraries.

           Various methods for synthesizing primers and probes are well-known in the art as are
methods for attaching labels to primers or probes. For example, it is a matter of routine to
synthesize desired nucleic acid primers or probes using conventional nucleotide
15   phosphoramidite chemistry and instruments available from Applied Biosystems, Inc., (Foster
City, CA), Dupont (Wilmington, DE), or Milligen (Bedford MA). Many methods have been
described for labeling oligonucleotides such as the primers or probes of the present invention.
Commercially available probe labeling kits include those from Amersham Life Science
(Arlington Heights, IL), Promega (Madison, WI), Enzo Biochemical (New York, NY) and
20   Clontech (Palo Alto, CA).

IV. Vectors and Host Cells
           The present invention provides vectors which include polynucleotides of the present
invention and host cells which are genetically engineered with vectors of the present
25   invention.
           a. Vectors and Expression Systems
           The present invention includes recombinant constructs comprising one or more of the
sequences as broadly described above. The constructs comprise a vector, such as a plasmid
or viral vector, into which a sequence of the invention has been inserted, in a forward or
30   reverse orientation. Such vectors include chromosomal, nonchromosomal and synthetic
DNA sequences from prokaryotic or eukaryotic sources. Large numbers of suitable plasmids
and vectors are known to those of skill in the art, and are commercially available. Vectors
which are particularly useful for cloning and expression in intermediate hosts include but are
not limited to: (a) Bacterial: pBR322 (ATCC 37017); pGEM (Promega Biotec, Madison,
35   WI), pUC, pSPORT1 and pProEx1 (Life Technologies, Gaithersburg, MD); pQE70, pQE60,
pQE-9 (Qiagen); pBs, phagescript, psiX174, pBluescript SK, pBsKS, pNH8a, pNH16a,

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pNH18a, pNH46a (Stratagene®, La Jolla, CA); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5, and pGEX4T (Pharmacia®, Piscataway, NJ); and (b) Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene®); pSVK3, pBPV, pMSG, pSVL (Pharmacia®); pcDNA3.1 (Invitrogen, Carlsbad, CA). Other appropriate cloning and expression vectors for use with
5   prokaryotic and eukaryotic hosts are described by Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Press, N.Y., 1982), which is hereby incorporated by reference. Generally however, any plasmid or vector may be used as long as it is replicable and viable in a host.

       In another embodiment, the construct is an expression vector which also comprises
10   regulatory sequences operably linked to the sequence of interest, to direct mRNA synthesis and polypeptide production. Regulatory sequences known to operate in prokaryotic and/or eukaryotic cells include inducible and non-inducible promoters for regulating mRNA transcription, ribosome binding sites for translation initiation, stop codons for translation termination and transcription terminators and/or polyadenylation signals. In addition, an
15   expression vector may include appropriate sequences for amplifying expression.

       Promoter regions may be selected from any desired gene. Particular named bacterial promoters include *lacZ*, *gpt*, lambda P_R, lambda P_L, *trc*, *trp*, *ermE* and its derivatives such as *ermEP*1 TGG, also known in the art as *ermE**, (Bibb, M. J., *et al.*, *Molecular Microbiology*, 14(3): 533-545 (1994)), *melCI*, and *actII* (C.M. Kao, *et al.*, *Science*, 265: 509-512 (1994)).
20   Eukaryotic promoters include cytomegalovirus (CMV) immediate early, herpes simplex virus (HSV) thymidine kinase, early and late SV40, LTRs from retroviruses, mouse metallothionein-I, prion protein and neuronal specific enolase (NSE). Selection of the appropriate promoter is well within the level of ordinary skill in the art. In addition, a recombinant expression vector will include an origin of replication and selectable marker
25   (such as a gene conferring resistance to an antibiotic (eg. neomycin, chloramphenicol, ampicillin, or thiostrepton) or a reporter gene (eg. luciferase)) which permit selection of stably transformed or transfected host cells.

       In any expression vector, a heterologous structural sequence (i.e. a polynucleotide of the present invention) is assembled in appropriate phase with translation initiation and
30   termination sequences. Optionally, the heterologous sequence will encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

       Eukaryotic expression vectors will also generally comprise an origin of replication, a suitable promoter operably linked to a sequence of interest and also any necessary translation
35   enhancing sequence, polyadenylation site, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for

example, SV40 origin, early promoter, enhancer, and polyadenylation sites may be used to provide the required genetic elements. Such vectors may also include an enhancer sequence to increase transcription of a gene. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription rate. Examples include

5   the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

       i. Vector construction

       The appropriate DNA sequence may be inserted into a vector by a variety of

10   procedures. Generally, site-specific DNA cleavage is performed by treating the DNA with suitable restriction enzymes under conditions which are generally specified by the manufacturer of these commercially available enzymes. Usually, about 1 microgram (µg) of plasmid or DNA sequence is cleaved by 1 unit of enzyme in about 20 microliters (µL) of buffer solution by incubation at 37°C for 1 to 2 hours. After incubation with the restriction

15   enzyme, protein can be removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved fragments may be separated using polyacrylamide or agarose gel electrophoresis, according to methods known by the routine practitioner. (See Maniatis *et al.*, *supra*).

       Ligations are performed using standard buffer and temperature conditions and with a

20   ligase (such as T4 DNA ligase) and ATP. Sticky end ligations require less ATP and less ligase than blunt end ligations. Vector fragments may be treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIAP) to remove the 5'-phosphate and thus prevent religation of the vector. Ligation mixtures are transformed into suitable cloning hosts such as *E. coli* and successful transformants selected by methods including

25   antibiotic resistance, and then screened for the correct construct.

       ii. Transformation/Transfection

       Transformation or transfection of an appropriate host with a construct of the invention, such that the host produces recombinant polypeptides, may also be performed in a variety of ways. For example, a construct may be introduced into a host cell by calcium

30   chloride or polyethylene glycol transformation, lithium chloride or calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. These and other methods for transforming/transfecting host cells are well known to routine practitioners (see L. Davis *et al.*, "Basic Methods in Molecular Biology", 2nd edition, Appleton and Lang, Paramount Publishing, East Norwalk, CT (1994) and D.A. Hopwood *et al.*, Genetic

35   Manipulation of *Streptomyces*: a laboratory manual, The John Innes Foundation, Norwich, England (1985)).

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b. Host Cells

In one embodiment, the present invention provides host cells containing recombinant
constructs as described below. In one aspect, a host cell may be an "intermediate" host
which is used to produce polynucleotides of the invention on a large-scale basis (for the
5   purpose of cloning and/or verifying recombinant polynucleotide sequences, for example) or
as a means to maintain such polynucleotide sequences over time (i.e. as maintenance or
storage strains). A "production" host is a host cell which is used to produce novel
polyketides. The host cell (either intermediate or production) can be a higher eukaryotic cell,
such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or a prokaryotic
10   cell, such as a bacterial cell. Lower eukaryotic and prokaryotic cells are preferred
intermediate and production hosts.

Representative examples of appropriate hosts include bacterial cells, such as *E. coli,
Bacillus subtilis, Saccharopolyspora erythraea, Streptomyces caelestis, Streptomyces
hygroscopicus, Streptomyces venezuelae*; and various other species within the genera
15   *Arthrobacter, Micromonospora, Nocardia, Pseudomonas, Streptomyces, Staphylococcus*, and
Saccharopolyspora, although others (of eukaryotic origin) may also be employed. Additional
representative examples of host cells are polyketide-producing microorganisms (as defined
above). The selection of an appropriate host is deemed to be within the scope of those skilled
in the art from the teachings provided herein.

20   Host cells are genetically engineered (transduced, transformed, transfected,
conjugated, or electroporated) with the vectors of this invention which may be a cloning
vector or an expression vector. The engineered host cells can be cultured in conventional
nutrient media modified as appropriate for activating promoters, selecting transformants, or
as a source of a biosynthetic substrate. The culture conditions, such as temperature, pH and
25   the like, are those previously used with the host cell selected for expression, and will be
apparent to the ordinarily skilled artisan.

V. Novel Polyketides and Methods of Making Novel Polyketides

The invention also provides novel polyketides, intermediate compounds thereof, and
30   methods for producing novel polyketides. The methods utilize the polyketide biosynthetic
genes from *Sac. erythraea* (i.e. the *eryA* genes) as well as those from other known polyketide-
producing microorganisms and/or putative polyketide-producing microorganisms (i.e. those
having nucleotide sequences which hybridize to known PKS sequences but whose polyketide
products are unknown).

35   The organization of *eryA* and the DEBS encoded therefrom (see FIG. 1 and FIG. 2)
have been described in co-pending U.S. application Serial No. 07/642,734, filed January 17,

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1991, which is incorporated herein by reference in its entirety. As FIG. 2 shows, DEBS is organized in modules, with each module being responsible for one condensation step through the action of the resident KS, AT and ACP domains within that module wherein an extender unit, methylmalonyl CoA, is added first to the starter unit, propionyl CoA, and then

5   successively to the growing acyl chain. The precise succession of the elongation steps is dictated by the order of the six modules: module 1 determines the first condensation; module 2, the second; module 3, the third, and so on until the sixth condensation step has occurred. In addition, the choice of extender unit that is incorporated into a growing polyketide chain at each condensation is determined, in whole or in part, by the AT domain within each module.

10   In the case of DEBS, the extender unit incorporated is always methylmalonate. Thus, as 6-deoxyerythronolide B grows through successive condensations, two carbons are added to the nascent chain and every other carbon, starting with the carbon corresponding to C-12 in the ring, carries a methyl group as a side chain.

           As also seen in FIG. 2, the processing of the growing carbon chain after each

15   condensation is determined by the information within each module. Thus, β-ketoreduction of the β-keto group generated by the condensation event takes place after each condensation step except the third, as determined by the presence of an active KR domain in each module except module 3, whereas dehydration and enoylreduction take place after the fourth condensation step, as determined by the presence of the DH and ER domains in module 4.

20   Once the polyketide chain is fully synthesized, it is released from the PKS through the action of the TE domain present at the end of module 6 and cyclizes to form the macrocyclic lactone 6-deoxyerythronolide B which is subsequently acted upon by a series of other enzymes, whose genes reside in the erythromycin cluster of the *Sac. erythraea* chromosome (see FIG. 1). As shown in FIG. 1, erythromycin carries methyl side chains at position 2, 4, 6, 8, 10 and

25   12, through the incorporation of methylmalonate as the extender unit at each step of synthesis of the polyketide moiety.

           In the present invention, novel polyketide molecules of a desired structure are produced by introducing specific genetic alterations into a PKS-encoding sequence in the genome of a polyketide-producing microorganism. Alteration of one or more genes or

30   fragments thereof may be generated through manipulation of genes residing exclusively within a species (i.e. intraspecies alterations), and include not only manipulations of genes within a single PKS cluster but also between different PKS clusters residing within a single strain (as is seen in *S. hygroscopicus*). Several examples of intraspecies alterations showing the manipulation of genes exclusively within a single PKS (namely, *eryA*) are described in

35   U.S. application Serial No. 07/624,734 cited *supra*. Alternatively, a gene or fragment thereof may be exchanged with a heterologous gene or gene fragment encoding one or more related

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domains from the PKS of a different polyketide-producing microorganism (interspecies alterations). Several examples of novel polyketides produced from exchange of heterologous genes are provided herein.

Whether the genetic manipulations are performed intraspecies or interspecies, three types of alterations to a PKS sequence may be carried out: (i) those which affect a module but do not cause the arrest of chain growth (Type I alterations); (ii) those which affect a single function in a module thereby causing the arrest of chain growth (Type II alterations); and (iii) those which affect an entire module (Type III alterations). In one embodiment, Type I alterations are produced by inactivation of domains that specify the functional groups and/or degree of oxidation found at specific ring positions in the native polyketide. Such domains typically include ß-ketoreductases, dehydratases and enoylreductases. For example, an allele corresponding to ß-ketoreductase of module 5 may be mutated by deleting a substantial portion of the DNA encoding the ß-ketoreductase (thereby producing an inactive domain) and used to replace the wild-type allele in the native strain. Such a transfer results in the production of the novel polyketide 5-oxo-5,6-dideoxy-3-∝-mycarosyl erythronolide B.

In an alternative embodiment, Type I alterations are generated by replacing at least one domain in a particular PKS with at least one related domain from the same or a second PKS. Such related domains may exist between different polyketide-producing microorganisms (such as for example, the AT domains of *Sac. erythraea, S. venezuelae, S. hygroscopicus,* and *S. caelestis*) or within a single species (as for example, the LigAT2 and rapAT1 domains in *S. hygroscopicus*).

Ways to identify polyketide synthases, their domains and the functional similarity of domains are well-known to those of ordinary skill in the art. For example, the PKS region of the chromosome of a polyketide or putative polyketide-producing microorganism may be identified by hybridizing with nucleic acid probes under conditions of low or high stringency. Hybridization under high stringency conditions is generally performed in a buffer consisting of 15 mM sodium chloride and 1.5 mM trisodium citrate (0.1 x SSC) with an incubation temperature of about 65°C (see for example, Maniatis, *et al. supra*). To detect more distantly related PKS genes, hybridization is performed under low stringency conditions which include lower temperature incubations and/or the presence of increased amounts of sodium chloride and trisodium citrate (Maniatis, *et al. supra*). Once identified, the chromosomal region may be isolated, cloned into a suitable vector and sequenced, using conventional methods or commercial sequencing kits such as Sequenase (US Biochemical Corp, Cleveland, OH). Methods for isolating and cloning chromosomal DNA are also well known in the art (Maniatis, *et al. supra*). An amino acid sequence may then be deduced from the DNA sequence and a comparison made of the unknown amino acid sequence to that of one or more

polypeptides involved in polyketide biosynthesis. Two amino acid sequences showing at least about 20% and more preferably about 25% identity and having conserved active site residues or motifs are considered to specify functionally similar or equivalent PKS domains. Having identified such domains, the number and composition of modules as well as the

5   arrangement of modules within particular ORFs can be determined.

In the case where the newly defined PKS produces a polyketide of known structure, the ß-carbonyl processing and types of side chain moieties and their positioning on the polyketide backbone can be correlated to specific domains within modules. Because modules are established linearly within ORFs, this correlation also allows one to determine the order

10   of modular activity (i.e. which module catalyzes which condensation step) in the PKS. For example, the ß-carbonyl processing and types of side chain moieties in the polyketide generates a pattern of chemical groups that can be correlated to a pattern of domains within an ORF. Based on the specific type of side chain moiety at a given carbon, one can then predict the particular substrate utilized by that module's AT domain.

15   In the case where the polyketide structure is unknown, theoretically, comparative sequence analysis alone may be used to predict the substrate specificity of an AT domain. To accomplish this, at least two and preferably, three or more sequences known or predicted to specify a particular substrate can be compared to determine one or more conserved or consensus motifs unique to that family of ATs. An unknown AT having such motifs can then

20   be assigned to a particular family.

Alternatively, comparative analyses can be performed using computer programs which group AT domains based on primary amino acid sequence similarity or phylogenetic relationships. For example, comparative analyses were made of the amino acid sequences of the AT domains in DEBS with corresponding AT domains in the PKS for rapamycin to

25   determine whether the extender unit used by a particular AT domain, (either malonate or methylmalonate), correlated with the degree of sequence identity between these domains. Rapamycin is a large polyketide that is assembled through 14 condensation events; the rapamycin PKS possesses 14 AT domains whose sequences were deduced from known nucleotide sequences (Aparicio *et al.* Gene 169:9-16 (1996)). Amino acid sequence

30   comparisons of the 14 AT domains of the rapamycin PKS with each other and with the 6 AT domains from DEBS, showed that the AT domains fell into two distinct groupings in which the rapamycin AT domains from modules 1, 3, 4, 6, 7, 10 and 13 clustered with the 6 erythromycin AT domains and the rapamycin AT domains in modules 2, 5, 8, 9, 11, 12 and 14 formed a separate cluster (Haydock et al. *FEBS Letts.* 374:246-248 (1995)). Examination

35   of the polyketide structure of rapamycin indicated that methyl side chains were at positions on the lactone ring corresponding to condensation steps 1, 3, 4, 6, 7, 10 and 13, which

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suggested that methylmalonate was used as the extender unit during synthesis of these
sections of the acyl chain; protons at the positions of the lactone ring corresponding to
condensations steps 2, 5, 8, 9, 11, 12 and 14 suggested that malonate was utilized as the
extender unit during synthesis of these sections. Two additional AT domains described

5   herein, ligAT2 and venAT, were also found to cluster with the putative malonate AT domains
from the rapamycin PKS (FIG. 3). Having predicted that AT domains from rap modules 2, 5,
8, 9, 11, 12 or 14, as well as ligAT2 and venAT, specify malonate as extender units, the DNA
encoding such domains could be isolated, cloned and used to replace the DNA encoding one
or more AT domains in a PKS such as DEBS, in order to generate novel polyketides.

10   The techniques for determining the amino acid sequence "similarity" are well-known
in the art. In general, when two or more polypeptides are aligned with one another, their
sequence similarity refers to the amino acids at corresponding positions within each
polypeptide sequence that are identical or possess similar chemical and/or physical properties
such as charge or hydrophobicity. A so-termed "percent similarity" then can be determined

15   between the compared polypeptide sequences. In general, the term "identity" refers to an
exact nucleotide to nucleotide or amino acid to amino acid correspondence at a given position
of two polynucleotides or polypeptide sequences, respectively. Two amino acid sequences
(or for that matter, two or more polynucleotide sequences) can be compared by determining
their "percent identity." The programs available in the Wisconsin Sequence Analysis

20   Package, Version 8 (available from Genetics Computer Group (GCG), Madison, WI), for
example, the GAP program, are capable of calculating both the identity between two
polynucleotides and the identity and similarity between two polypeptide sequences,
respectively. Other programs for calculating and displaying similarity between sequences are
known in the art. For example, the Growtree program (GCG, Madison, WI) creates a

25   phylogenetic tree wherein the most closely related sequences are clustered and joined by the
shortest lines. This tree is derived from a matrix created by the program Distances (GCG,
Madison, WI) which calculates pairwise relationships within a group of aligned sequences.

           In a preferred embodiment, novel polyketide molecules of desired structure are
produced by the replacement of at least one AT domain-encoding fragment of DNA of the

30   Sac. erythraea chromosome with at least one heterologous AT domain-encoding fragment of
DNA from another PKS cluster to yield novel polyketide compounds which are derivatives of
6-deoxyerythronolide B, erythronolide B, 3-α-L-mycarosylerythronolide B, or erythromycins
A, B, C and D. Such derivatives are compounds wherein methyl (-Me) side chains at one or
more positions of the macrocylic lactone ring are replaced by substituents independently

35   selected from the group consisting of (a) -H; (b) ethyl group (-Et); (c) hydroxyl group (-OH)
and (d) allyl group (-Al). In a particularly preferred embodiment, a method is provided for

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the genetic modification of erythromycin-producing microorganisms which enables them to produce the novel compounds 12-desmethyl-12-deoxyerythromycin A, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, or 6-desmethyl-6-ethylerythromycin A. The compounds 12-desmethyl-12-deoxyerythromycin A, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, and 6-desmethyl-6-ethylerythromycin A are represented by the structural formulae:
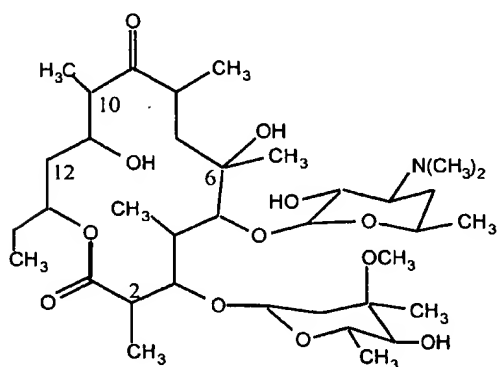
12-desmethyl-12-deoxyerythromycin A (I)
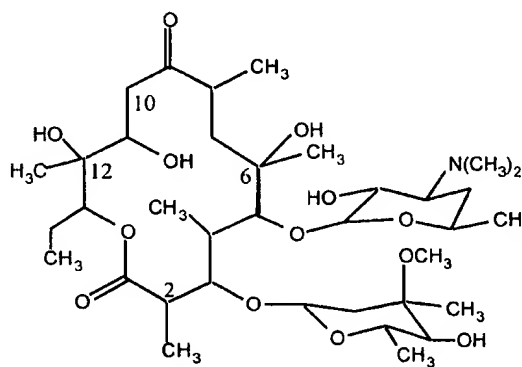
10-desmethylerythromycin A (II)
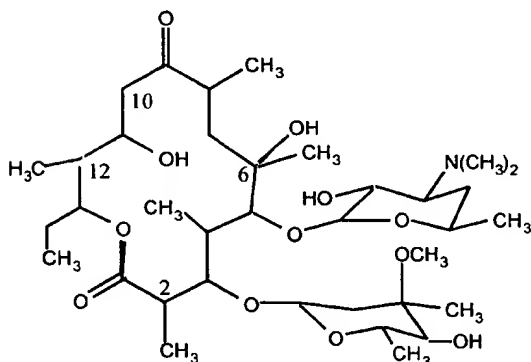
10-desmethyl-12-deoxyerythromycin A (III)
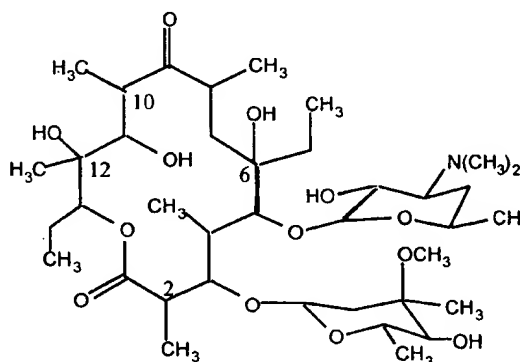
6-desmethyl-6-ethylerythromycin A (IV)

The general scheme for producing such polyketides is outlined in FIG. 4a and FIG. 4b. In the preferred embodiment, heterologous DNA fragments encoding related AT domains are introduced into the *Sac. erythraea* chromosome by a two-step method termed gene replacement.

In the first step of gene replacement, an integration vector is constructed through a multi-step cloning approach that places a heterologous gene or fragment thereof between two segments of DNA having sequences which are identical to those that immediately border (on each side) the resident polynucleotide sequence to be replaced. Construction of such a vector

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may be achieved by any means known to those of ordinary skill in art. For example, nucleotide sequences which flank the gene to be replaced can be generated by PCR amplification using chromosomal DNA as template and primers which hybridize to the chromosomal sequences immediately upstream and downstream of the flanking sequences of

5   interest. The length of the flanking sequences is not critical to the practice of the invention but preferably is about 20-5000 base pairs (bp), more preferably about 100-5000 bp, and even more preferably about 500-5000 bp. A most preferred length of flanking sequence is about 750-1500 bp. Primers used for such amplifications may also comprise convenient restriction sites to facilitate cloning of the amplified sequences into suitable preparative vectors, to

10   facilitate insertion of the heterologous sequence of interest between the flanking sequences and/or to facilitate subcloning of the entire group of sequences (5'-flanking region/heterologous polynucleotide sequence of interest/flanking region-3') into suitable vectors for integration. The desired heterologous polynucleotide sequences may be generated in a like manner.

15   The integration vectors are constructed to also comprise a fragment of DNA containing at least one origin of replication that is functional in an intermediate host but is non-functional or poorly functional in the production host. The vectors further comprise one or more fragments of DNA conferring resistance to an antibiotic, of which at least one functions in the intermediate host and at least one functions in the production host. Preferred

20   integration vectors comprise the ColE1 and pIJ101 origins of replication, as found in plasmid pCS5 (J. Vara *et al.*, *J. Bacteriol.* 171:5872-5881 (1989)). A particularly preferred vector carries a DNA fragment conferring resistance to thiostrepton and ampicillin. However, those skilled in the art understand that the particular antibiotic resistance genes and origins of replication identified above are necessary only inasmuch as they allow for the generation and

25   selection of the desired recombinant plasmids and host cells. Other markers and origins of replication may also be used in the practice of the invention.

When the resident domains of a PKS are functional components of large multifunctional polypeptides, care must be taken in the construction of the integration plasmid so that the heterologous DNA fragment encoding the heterologous AT domain is

30   positioned in the correct orientation and reading frame to its flanking DNA segments so that upon translation from the beginning of the coding sequence, an enzymatically functional protein is produced. The correct positioning becomes immediately apparent from knowledge of the nucleotide sequences of the host PKS genes and the heterologous genes used for gene replacement.

35   In the second step, each of the integration vectors carrying a related gene or fragment thereof is independently introduced into a host strain and recombination between each of the

genomic fragments in the integration plasmid and its corresponding homologous fragment in the host strain chromosome is allowed to occur. This procedure results in the exchange of the resident AT-encoding DNA in the chromosome for its heterologous counterpart. The general scheme for gene replacement by homologous recombination is outlined in FIG. 5.

5   Procedures to introduce DNA into polyketide-producing microorganisms and to facilitate homologous recombination are described herein. However, those skilled in the art understand that alternative procedures for introducing DNA into a polyketide-producing microorganism, such as electroporation, transduction, or conjugation, are well known and may also be used in the practice of the invention. Procedures for cultivating polyketide-

10   producing microorganisms, as well as methods to recover novel polyketides produced from modified strains, to purify such compounds and to confirm the identity of those compounds (such as by mass spectrometry or NMR) are well-known to those of ordinary skill in the art.

         Although the present invention is described in the Examples that follow in terms of preferred embodiments, they are not to be regarded as limiting the scope of the invention.

15   The descriptions that follow serve to illustrate the principles and methodologies involved in creating novel derivatives of erythromycin. Whereas the examples below describe the replacement of the *Sac. erythraea* AT1, AT2, and AT4-encoding DNA fragments with a heterologous DNA fragment which encodes either an AT domain that specifies incorporation of malonate (malonate-AT) or an AT domain that specifies incorporation of ethylmalonate

20   (ethylmalonate-AT), those skilled in the art understand that one or more fragments of heterologous DNA encoding malonate, ethylmalonate, allylmalonate, and/or hydroxymalonate (tartronate)-AT domains can be used to replace the other AT-encoding DNA fragments of the erythromycin PKS in *Sac. erythraea* to result in the production of other novel erythromycin derivatives. For example, novel erythromycins produced when

25   resident AT-encoding DNA fragments in the erythromycin PKS (eryPKS) are independently replaced with heterologous DNA fragments specifying malonate and/or ethylmalonate as the extender unit are shown in Table 1.

         In particular, those skilled in the art understand that following the methods described herein for replacement of a single resident AT-encoding DNA fragment in the eryPKS,

30   replacements of two resident AT-encoding DNA fragments with heterologous DNA fragments (specifying malonate, ethylmalonate, allylmalonate, and/or hydroxymalonate -AT domains) in stepwise fashion are also possible and result in the formation of novel disubstituted erythromycins. Similarly, trisubstituted erythromycins, tetrasubstituted erythromycins, pentasubstituted erythromycins and hexasubstituted erythromycins can also

35   be made by replacement of three, four, five and six resident AT-encoding DNA fragments in the eryPKS, respectively, with heterologous AT-encoding DNA fragments as described

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herein. Therefore, all substitutions of AT-encoding DNA fragments in the eryPKS with heterologous AT-encoding DNA fragments (yielding all varieties of proton, ethyl, allyl, and hydroxyl substituted erythromycin derivatives) are within the scope of the present invention. Examples of compounds produced by such replacements include but are not limited to those shown in Table 1 below.

Table 1

Structures from Changes at Side Chain Positions
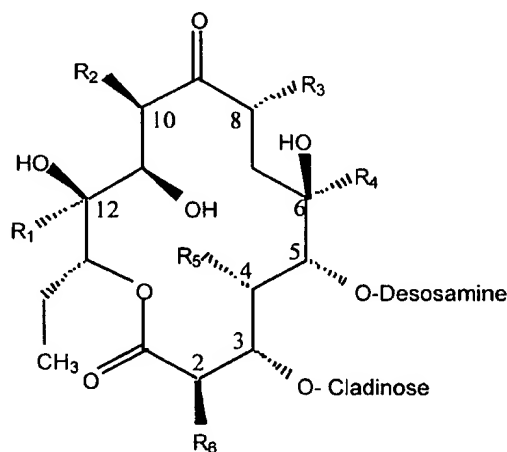
A. Single Changes

R_1	R_2	R_3	R_4	R_5	R_6	Name
H	Me	Me	Me	Me	Me	12-Desmethylerythromycin A
Et	Me	Me	Me	Me	Me	12-Desmethyl-12-ethylerythromycin A
Me	H	Me	Me	Me	Me	10-Desmethylerythromycin A
Me	Et	Me	Me	Me	Me	10-Desmethyl-10-ethylerythromycin A
Me	Me	H	Me	Me	Me	8-Desmethylerythromycin A
Me	Me	Et	Me	Me	Me	8-Desmethyl-8-ethylerythromycin A
Me	Me	Me	H	Me	Me	6-Desmethylerythromycin A
Me	Me	Me	Et	Me	Me	6-Desmethyl-6-ethylerythromycin A
Me	Me	Me	Me	H	Me	4-Desmethylerythromycin A
Me	Me	Me	Me	Et	Me	4-Desmethyl-4-ethylerythromycin A
Me	Me	Me	Me	Me	H	2-Desmethylerythromycin A
Me	Me	Me	Me	Me	Et	2-Desmethyl-2-ethylerythromycin A

B. Two Changes

R_1	R_2	R_3	R_4	R_5	R_6	Name
H	Me	Me	Me	Me	Et	2,12-Didesmethyl-2-ethylerythromycin A
H	Me	Me	Me	Et	Me	4,12-Didesmethyl-4-ethylerythromycin A
H	Me	Me	Et	Me	Me	6,12-Didesmethyl-6-ethylerythromycin A
H	Me	Et	Me	Me	Me	8,12-Didesmethyl-8-ethylerythromycin A
H	Et	Me	Me	ME	Me	10,12-Didesmethyl-10-ethylerythromycin A
H	Me	Me	Me	Me	H	2,12-Didesmethylerythromycin A
H	Me	Me	Me	H	Me	4,12-Didesmethylerythromycin A
H	Me	Me	H	Me	Me	6,12-Didesmethylerythromycin A
H	Me	H	Me	Me	Me	8,12-Didesmethylerythromycin A

H	H	Me	Me	Me	Me	10,12-Didesmethylerythromycin A
Me	H	Me	Me	Me	Et	2,10-Didesmethyl-2-ethylerythromycin A
Me	H	Me	Me	Et	Me	4,10-Didesmethyl-4-ethylerythromycin A
Me	H	Me	Et	Me	Me	6,10-Didesmethyl-6-ethylerythromycin A
Me	H	Et	Me	Me	Me	8,10-Didesmethyl-8-ethylerythromycin A
Me	H	Me	Me	Me	H	2,10-Didesmethylerythromycin A
Me	H	Me	Me	H	Me	4,10-Didesmethylerythromycin A
Me	H	Me	H	Me	Me	6,10-Didesmethylerythromycin A
Me	H	H	Me	Me	Me	8,10-Didesmethylerythromycin A
Me	Me	H	Me	Me	Et	2,8-Didesmethyl-2-ethylerythromycin A
Me	Me	H	Me	Et	Me	4,8-Didesmethyl-4-ethylerythromycin A
Me	Me	H	Et	Me	Me	6,8-Didesmethyl-6-ethylerythromycin A
Me	Me	H	Me	Me	H	2,8-Didesmethylerythromycin A
Me	Me	H	Me	H	Me	4,8-Didesmethylerythromycin A
Me	Me	H	H	Me	Me	6,8-Didesmethylerythromycin A
Me	Me	Me	H	Me	Et	2,6-Didesmethyl-2-ethylerythromycin A
Me	Me	Me	H	Et	Me	4,6-Didesmethyl-4-ethylerythromycin A
Me	Me	Me	H	Me	H	2,6-Didesmethylerythromycin A
Me	Me	Me	H	H	Me	4,6-Didesmethylerythromycin A
Me	Me	Me	Me	H	Et	2,4,-Didesmethyl-2-ethylerythromycin A
Me	Me	Me	Me	H	H	2,4,-Didesmethylerythromycin A
Et	Me	Me	Me	Me	Et	2,12-Didesmethyl-2,12-diethylerythromycin A
Et	Me	Me	Me	Et	Me	4,12-Didesmethyl-4,12-diethylerythromycin A
Et	Me	Me	Et	Me	Me	6,12-Didesmethyl-6,12-diethylerythromycin A
Et	Me	Et	Me	Me	Me	8,12-Didesmethyl-8,12-diethylerythromycin A
Et	Et	Me	Me	Me	Me	10,12-Didesmethyl-10,12-diethylerythromycin A
Et	Me	Me	Me	Me	H	2,12-Didesmethyl-12-ethylerythromycin A
Et	Me	Me	Me	H	Me	4,12-Didesmethyl-12-ethylerythromycin A
Et	Me	Me	H	Me	Me	6,12-Didesmethyl-12-ethylerythromycin A
Et	Me	H	Me	Me	Me	8,12-Didesmethyl-12-ethylerythromycin A
Et	H	Me	Me	Me	Me	10,12-Didesmethyl-12-ethylerythromycin A
Me	Et	Me	Me	Me	Et	2,10-Didesmethyl-2,10-diethylerythromycin A
Me	Et	Me	Me	Et	Me	4,10-Didesmethyl-4,10-diethylerythromycin A
Me	Et	Me	Et	Me	Me	6,10-Didesmethyl-6,10-diethylerythromycin A
Me	Et	Et	Me	Me	Me	8,10-Didesmethyl-8,10-diethylerythromycin A
Me	Et	Me	Me	Me	H	2,10-Didesmethyl-10-ethylerythromycin A
Me	Et	Me	Me	H	Me	4,10-Didesmethyl-10-ethylerythromycin A
Me	Et	Me	H	Me	Me	6,10-Didesmethyl-10-ethylerythromycin A
Me	Et	H	Me	Me	Me	8,10-Didesmethyl-10-ethylerythromycin A
Me	Me	Et	Me	Me	Et	2,8-Didesmethyl-2,8-diethylerythromycin A
Me	Me	Et	Me	Et	Me	4,8-Didesmethyl-4,8-diethylerythromycin A
Me	Me	Et	Et	Me	Me	6,8-Didesmethyl-6,8-diethylerythromycin A
Me	Me	Et	Me	Me	H	2,8-Didesmethyl-8-ethylerythromycin A
Me	Me	Et	Me	H	Me	4,8-Didesmethyl-8-ethylerythromycin
Me	Me	Et	H	Me	Me	6,8-Didesmethyl-8-ethylerythromycin
Me	Me	Me	Et	Me	Et	2,6-Didesmethyl-2,6-diethylerythromycin A
Me	Me	Me	Et	Et	Me	4,6-Didesmethyl-4,6-diethylerythromycin A
Me	Me	Me	Et	Me	H	2,6-Didesmethyl-6-ethylerythromycin A
Me	Me	Me	Et	H	Me	4,6-Didesmethyl-6-ethylerythromycin
Me	Me	Me	Me	Et	Et	2,4-Didesmethyl-2,4-diethylerythromycin A
Me	Me	Me	Me	Et	H	2,4-Didesmethyl-4-ethylerythromycin A

C. Three Changes

H	H	Me	Me	Me	Et	2,10,12-Tridesmethyl-2-Ethylerythromycin A

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H	H	Me	Me	Me	H	2,10,12-Tridesmethylerythromycin A
H	H	Me	Me	Et	Me	4,10,12-Tridesmethyl-4-Ethylerythromycin A
H	H	Me	Me	H	Me	4,10,12-Tridesmethylerythromycin A
H	H	Me	Et	Me	Me	6,10,12-Tridesmethyl-6-Ethylerythromycin A
H	H	Me	H	Me	Me	6,10,12-Tridesmethylerythromycin A
H	H	Et	Me	Me	Me	8,10,12-Tridesmethyl-8-ethylerythromycin A
H	H	H	Me	Me	Me	8,10,12-Tridesmethylerythromycin A
Et	H	Me	Me	Me	Et	2,10,12-Tridesmethyl-2,12,-diethylerythromycin A
Et	H	Me	Me	Me	H	2,10,12-Tridesmethyl-12-ethylerythromycin A
Et	H	Me	Me	Et	Me	4,10,12-Tridesmethyl-4,12-diethylerythromycin A
Et	H	Me	Me	H	Me	4,10,12-Tridesmethyl-12-ethylerythromycin A
Et	H	Me	Et	Me	Me	6,10,12-Tridesmethyl-6,12-diethylerythromycin A
Et	H	Me	H	Me	Me	6,10,12-Tridesmethyl-12-ethylerythromycin A
Et	H	Et	Me	Me	Me	8,10,12-Tridesmethyl-8,12-diethylerythromycin A
Et	H	H	Me	Me	Me	8,10,12 Tridesmethyl-12-ethylerythromycin A
H	Et	Me	Me	Me	Et	2,10,12-Tridesmethyl-2,10-diethylerythromycin A
H	Et	Me	Me	Me	H	2,10,12-Tridesmethyl-10-ethylerythromycin A
H	Et	Me	Me	Et	Me	4,10,12-Tridesmethyl-4,10-diethylerythromycin A
H	Et	Me	Me	H	Me	4,10,12-Tridesmethyl-10-ethylerythromycin A
H	Et	Me	Et	Me	Me	6,10,12-Tridesmethyl-6,10-diethylerythromycin A
H	Et	Me	H	Me	Me	6,10,12-Tridesmethyl-10-ethylerythromycin A
H	Et	Et	Me	Me	Me	8,10,12-Tridesmethyl-8,10-diethylerythromycin A
H	Et	H	Me	Me	Me	8,10,12-Tridesmethyl-10-ethylerythromycin A
Et	Et	Me	Me	Me	Et	2,10,12-Tridesmethyl-2,10,12-triethylerythromycin A
Et	Et	Me	Me	Me	H	2,10,12-Tridesmethyl-10,12-diethylerythromycin A
Et	Et	Me	Me	Et	Me	4,10,12-Tridesmethyl-4,10,12-triethylerythromycin A
Et	Et	Me	Me	H	Me	4,10,12-Tridesmethyl-10,12,-diethylerythromycin A
Et	Et	Me	Et	Me	Me	6,10,12-Tridesmethyl-6,10,12-triethylerythromycin A
Et	Et	Me	H	Me	Me	6,10,12-Tridesmethyl-10,12-diethylerythromycin A
Et	Et	Et	Me	Me	Me	8,10,12-Tridesmethyl-8,10,12-triethylerythromycin A
Et	Et	H	Me	Me	Me	8,10,12-Tridesmethyl-10,12-diethylerythromycin A
H	Me	H	Me	Me	Et	2,8,12-Tridesmethyl-2-ethylerythromycin A
H	Me	H	Me	Me	H	2,8,12-Tridesmethylerythromycin A
H	Me	H	Me	Et	Me	4,8,12-Tridesmethyl-4- ethylerythromycin A
H	Me	H	Me	H	Me	4,8,12-Tridesmethylerythromycin A
H	Me	H	Et	Me	Me	6,8,12-Tridesmethyl-6- ethylerythromycin A
H	Me	H	H	Me	Me	6,8,12-Tridesmethylerythromycin A
Et	Me	H	Me	Me	Et	2,8,12-Tridesmethyl-2,12-diethylerythromycin A
Et	Me	H	Me	Me	H	2,8,12-Tridesmethyl-12-ethylerythromycin A
Et	Me	H	Me	Et	Me	4,8,12-Tridesmethyl-4,12-diethylerythromycin A
Et	Me	H	Me	H	Me	4,8,12-Tridesmethyl-12-ethylerythromycin A
Et	Me	H	Et	Me	Me	6,8,12-Tridesmethyl-6,12-diethylerythromycin A
Et	Me	H	H	Me	Me	6,8,12-Tridesmethyl-12-ethylerythromycin A
H	Me	Et	Me	Me	Et	2,8,12-Tridesmethyl-2,8-diethylerythromycin A
H	Me	Et	Me	Me	H	2,8,12-Tridesmethyl-8-ethylerythromycin A
H	Me	Et	Me	Et	Me	4,8,12-Tridesmethyl-4,8-diethylerythromycin A
H	Me	Et	Me	H	Me	4,8,12-Tridesmethyl-8-ethylerythromycin A
H	Me	Et	Et	Me	Me	6,8,12-Tridesmethyl-6,8-diethylerythromycin A
H	Me	Et	H	Me	Me	6,8,12-Tridesmethyl-8-ethylerythromycin A
Et	Me	Et	Me	Me	Et	2,8,12-Tridesmethyl-2,8,12-triethylerythromycin A
Et	Me	Et	Me	Me	H	2,8,12-Tridesmethyl-8,12-diethylerythromycin A
Et	Me	Et	Me	Et	Me	4,8,12-Tridesmethyl-4,8,12-triethylerythromycin A
Et	Me	Et	Me	H	Me	4,8,12-Tridesmethyl-8,12-diethylerythromycin A
Et	Me	Et	Et	Me	Me	6,8,12-Tridesmethyl-6,8,12-triethylerythromycin A

Et	Me	Et	H	Mc	Mc	6,8,12-Tridesmethyl-8,12-diethylerythromycin A
H	Me	Me	H	Me	Et	2,6,12-Tridesmethyl-2-ethylerythromycin A
H	Me	Me	H	Me	H	2,6,12-Tridesmethylerythromycin A
H	Me	Me	H	Et	Me	4,6,12-Tridesmethyl-4-ethylerythromycin A
H	Me	Me	H	H	Me	4,6,12-Tridesmethylerythromycin A
Et	Me	Me	H	Me	Et	2,6,12-Tridesmethyl-2,12-diethylerythromycin A
Et	Me	Mc	H	Mc	H	2,6,12-Tridcsmethyl-12-ethylerythromycin A
Et	Me	Me	H	Et	Me	4,6,12-Tridesmethyl-4,12- diethylerythromycin A
Et	Me	Me	H	H	Me	4,6,12-Tridesmethyl-12-ethylerythromycin A
H	Me	Me	Et	Me	Et	2,6,12-Tridesmethyl-2,6-diethylcrythromycin A
H	Me	Me	Et	Me	H	2,6,12-Tridesmethyl-6,-ethylerythromycin A
H	Me	Me	Et	Et	Me	4,6,12-Tridesmethyl-4,6-diethylerythromycin A
H	Me	Me	Et	·H	Me	4,6,12-Tridesmethyl-6-ethylerythromycin A
Et	Me	Me	Et	Me	Et	2,6,12-Tridesmethyl-2,6,12-triethylerythromycin A
Et	Me	Me	Et	Me	H	2,6,12-Tridesmethyl-6,12-diethylerythromycin A
Et	Me	Me	Et	Et	Me	4,6,12-Tridesmethyl-4,6,12-triethylerythromycin A
Et	Me	Me	Et	H	Me	4,6,12-Tridesmethyl-6,12-diethylerythromycin A
H	Me	Me	Me	H	Et	2,4,12-Tridesmethyl-2-ethylerythromycin A
H	Me	Me	Me	H	H	2,4,12-Tridesmethylerythromycin A
Et	Me	Me	Me	H	Et	2,4,12-Tridesmethyl-2,12-diethylerythromycin A
Et	Me	Me	Me	H	H	2,4,12-Tridesmethyl-12-ethylerythromycin A
H	Me	Me	Me	Et	Et	2,4,12-Tridesmethyl-2,4-diethylerythromycin A
H	Me	Me	Me	Et	H	2,4,12-Tridesmethyl-4-ethylerythromycin A
Et	Me	Me	Me	Et	Et	2,4,12-Tridesmethyl-2,4,12-triethylerythromycin A
Et	Me	Me	Me	Et	H	2,4,12-Tridesmethyl-2,12-diethylerythromycin A
Me	H	H	Me	Me	Et	2,8,10-Tridesmethyl-2-ethylerythromycin A
Me	H	H	Me	Me	H	2,8,10-Tridesmethylerythromycin A
Me	H	H	Me	Et	Me	4,8,10-Tridesmethyl-4-ethylerythromycin A
Me	H	H	Mc	H	Me	4,8,10-Tridesmethylerythromycin A
Me	H	H	Et	Me	Me	6,8,10-Tridesmethyl-6-ethylerythromycin A
Me	H	H	H	Me	Me	6,8,10-Tridesmethylerythromycin A
Me	Et	H	Mc	Me	Et	2,8,10-Tridesmethyl-2,10-diethylerythromycin A
Me	Et	H	Me	Me	H	2,8,10-Tridesmethyl-10-ethylerythromycin A
Me	Et	H	Me	Et	Me	4,8,10-Tridesmethyl-4,10-diethylerythromycin A
Me	Et	H	Me	H	Me	4,8,10-Tridesmethyl-10-ethylerythromycin A
Me	Et	H	Et	Me	Me	6,8,10-Tridesmethyl-6,10-diethylerythromycin A
Me	Et	H	H	Me	Me	6,8,10-Tridesmethyl-10-ethylerythromycin A
Me	H	Et	Me	Me	Et	2,8,10-Tridesmethyl-2,8-diethylerythromycin A
Me	H	Et	Me	Me	H	2,8,10-Tridesmethyl-8-ethylerythromycin A
Me	H	Et	Me	Et	Me	4,8,10-Tridesmethyl-4,8-diethylerythromycin A
Me	H	Et	Me	H	Me	4,8,10-Tridesmethyl-8-ethylerythromycin A
Me	H	Et	Et	Me	Mc	6,8,10-Tridesmethyl-6,8-diethylerythromycin A
Me	H	Et	H	Me	Me	6,8,10-Tridesmethyl-8-ethylerythromycin A
Me	Et	Et	Me	Me	Et	2,8,10-Tridesmethyl-2,8,10-triethylerythromycin A
Me	Et	Et	Me	Me	H	2,8,10-Tridesmethyl-8,10-diethylerythromycin A
Me	Et	Et	Me	Et	Me	4,8,10-Tridesmethyl-4,8,10-triethylerythromycin A
Me	Et	Et	Me	H	Me	4,8,10-Tridesmethyl-8-10-diethylerythromycin A
Me	Et	Et	Et	Me	Me	6,8,10-Tridesmethyl-6,8,10-triethylerythromycin A
Me	Et	Et	H	Me	Me	6,8,10-Tridesmethyl-8,10-diethylerythromycin A
Me	H	Me	H	Me	Et	2,6,10-Tridesmethyl-2-ethylerythromycin A
Me	H	Me	H	Me	H	2,6,10-Tridesmethylerythromycin A.
Me	H	Me	H	Et	Me	4,6,10-Tridesmethyl-4-ethylerythromycin A
Me	H	Me	H	H	Me	4,6,10-Tridesmethylerythromycin A
Me	Et	Me	H	Me	Et	2,6,10-Tridesmethyl-2,1--diethylerythromycin A

Me	Et	Me	H	Me	H	2,6,10-Tridesmethyl-10-ethylerythromycin A
Me	Et	Me	H	Et	Me	4,6,10-Tridesmethyl-4,10-diethylerythromycin A
Me	Et	Me	H	H	Me	4,6,10-Tridesmethyl-10-ethylerythromycin A
Me	H	Me	Et	Mc	Et	2,6,10-Tridesmethyl-2,6-diethylerythromycin A
Me	H	Me	Et	Me	H	2,6,10-Tridesmethyl-6-ethylerythromycin A
Me	H	Me	Et	El	Me	4,6,10-Tridesmethyl-4,6,diethylerythromycin A
Me	II	Me	Et	H	Me	4,6,10-Tridesmethyl-6-ethylerythromycin A
Me	Et	Me	Et	Me	Et	2,6,10-Tridesmethyl-2,6,10-triethylerythromycin A
Me	Et	Me	Et	Me	H	2,6,10-Tridesmethyl-6,10-diethylerythromycin A
Me	Et	Me	Et	Et	Me	4,6,10-Tridesmethyl-4,6,10-triethylerythromycin A
Me	Et	Me	Et	H	Me	4,6,10-Tridesmethyl-6,10-diethylerythromycin A
Me	H	Me	Me	H	Et	2,4,10-Tridesmethyl-2-ethylerythromycin A
Me	H	Me	Me	H	H	2,4,10-Tridesmethylerythromycin A
Me	Et	Me	Me	H	Et	2,4,10-Tridesmethyl-2,10-diethylerythromycin A
Me	Et	Me	Me	H	H	2,4,10-Tridesmethyl-10-ethylerythromycin A
Me	H	Me	Me	Et	Et	2,4,10-Tridesmethyl-2,4-diethylerythromycin A
Me	H	Me	Me	Et	H	2,4,10-Tridesmethyl-4-ethylerythromycin A
Me	Et	Me	Me	Et	Et	2,4,10-Tridesmethyl-2,4,10-triethylerythromycin A
Me	Et	Me	Me	Et	H	2,4,10-Tridesmethyl-4,10-diethylerythromycin A
Me	Me	H	H	Me	Et	2,6,8-Tridesmethyl-2-ethylerythromycin A
Me	Me	H	H	Me	H	2,6,8-Tridesmethylerythromycin A
Me	Me	H	H	Et	Me	4,6,8-Tridesmethyl-4-ethylerythromycin A
Me	Me	H	H	H	Me	4,6,8-Tridesmethylerythromycin A
Me	Me	Et	H	Me	Et	2,6,8-Tridesmethyl-2,8-diethylerythromycin A
Mc	Me	Et	H	Me	H	2,6,8-Tridesmethyl-8-ethylerythromycin A
Me	Me	Et	H	Et	Mc	4,6,8-Tridesmethyl-4,8-diethylerythromycin A
Me	Me	Et	H	H	Me	4,6,8-Tridesmethyl-8-ethylerythromycin A
Me	Me	H	Et	Me	Et	2,6,8-Tridesmethyl-2,6-diethylerythromycin A
Me	Me	H	Et	Me	H	2,6,8-Tridesmethyl-6-ethylerythromycin A
Me	Me	H	Et	Et	Me	4,6,8-Tridesmethyl-4,6-diethylerythromycin A
Mc	Me	H	Et	H	Me	4,6,8-Tridesmethyl-6-ethylerythromycin A
Me	Me	Et	Et	Me	Et	2,6,8-Tridesmethyl-2,6,8-triethylerythromycin A
Me	Me	Et	Et	Me	H	2,6,8-Tridesmethyl-6,8-diethylerythromycin A
Me	Me	Et	Et	Et	Me	4,6,8-Tridesmethyl-4,6,8-triethylerythromycin A
Me	Me	Et	Et	H	Me	4,6,8-Tridesmethyl-6,8-triethylerythromycin A
Me	Me	H	Me	H	Et	2,4,8- Tridesmethyl-2-ethylerythromycin A
Me	Me	H	Me	H	H	2,4,8-Tridesmethylerythromycin A
Me	Me	Et	Me	H	Et	2,4,8-Tridesmethyl-2,8-diethylerythromycin A
Me	Me	Et	Me	H	H	2,4,8-Tridesmethyl-8-ethylerythromycin A
Me	Me	H	Me	Et	Et	2,4,8-Tridesmethyl-2,4-diiethylerythromycin A
Me	Me	H	Me	Et	H	2,4,8-Tridesmethyl-4-ethylerythromycin A
Me	Me	Et	Me	Et	Et	2,4,8-Tridesmethyl-2,4,8-triethylerythromycin A
Me	Me	Et	Me	Et	H	2,4,8-Tridesmethyl-4,8-diethylerythromycin A
Mc	Me	Me	H	H	Et	2,4,6-Tridesmethyl-2-ethylerythromycin A
Me	Me	Me	H	H	H	2,4,6-Tridesmethylerythromycin A
Me	Me	Me	Et	H	Et	2,4,6-Tridesmethyl-2,6-diethylerythromycin A
Me	Me	Me	Et	H	H	2,4,6-Tridesmethyl-6-ethyl erythromycin A
Me	Me	Me	H	Et	Et	2,4,6-Tridesmethyl-2,4-diethyl erythromycin A
Me	Me	Me	H	Et	H	2,4,6-Tridesmethyl-4-ethyl erythromycin A
Me	Me	Me	Et	Et	Et	2,4,6-Tridesmethyl-2,4,6-triethyl erythromycin A
Me	Me	Me	Et	Et	H	2,4,6-Tridesmethyl-4,6-diethyl erythromycin A

D. Four Changes

H	H	H	Me	Me	Et	2,8,10,12-Tetradesmethyl-2-ethylerythromycin A

H	H	H	Me	Me	H	2,8,10,12-Tetradesmethylerythromycin A
H	H	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4-ethylerythromycin A
H	H	H	Mc	H	Me	4,8,10,12-Tetradesmethylerythromycin A
H	H	H	Et	Me	Mc	6,8,10,12-Tetradesmethyl-6-ethylerythromycin A
H	H	H	H	Me	Me	6,8,10,12-Tetradesmethylerythromycin A
H	Et	H	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,10-diethylerythromycin A
H	Et	H	Mc	Mc	H	2,6,10,12-Tetradesmethyl-10-ethylerythromycin A
H	Et	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,10-diethylerythromycin A
H	Et	H	Me	H	Me	4,8,10,12-Tetradesmethyl-10-ethylerythromycin A
H	Et	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,10-diethylerythromycin A
H	Et	H	H	Me	Me	6,8,10,12-Tetradesmethyl-10-ethylerythromycin A
H	H	Et	Me	Me	Et	2,8,10,12-Tetradesmethyl-2,8-diethylerythromycin A
H	H	Et	Me	Me	H	2,8,10,12-Tetradesmethyl-8-ethylerythromycin A
H	H	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8-diethylerythromycin A
H	H	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8-ethylerythromycin A
H	H	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8-diethylerythromycin A
H	H	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8-ethylerythromycin A
H	Et	Et	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,8,10-triethylerythromycin A
H	Et	Et	Me	Me	H	2,6,10,12-Tetradesmethyl-8,10-diethylerythromycin A
H	Et	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8,10-triethylerythromycin A
H	Et	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8,10-diethylerythromycin A
H	Et	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8,10-triethylerythromycin A
H	Et	Et	H	Me	Mc	6,8,10,12-Tetradesmethyl-8,10-diethylerythromycin A
Et	H	H	Me	Me	Et	2,8,10,12-Tetradesmethyl-2,12-diethylerythromycin A
Et	H	H	Me	Me	H	2,8,10,12-Tetradesmethyl-12-ethylerythromycin A
Et	H	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,12-diethylerythromycin A
Et	H	H	Me	H	Me	4,8,10,12-Tetradesmethyl-12-ethylerythromycin A
Et	H	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,12-diethylerythromycin A
Et	H	H	H	Me	Me	6,8,10,12-Tetradesmethyl-12-ethylerythromycin A
Et	Et	H	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,10,12-triethylerythromycin A
Et	Et	H	Me	Me	H	2,6,10,12-Tetradesmethyl-10,12-diethylerythromycin A
Et	Et	H	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,10,12-triethylerythromycin A
Et	Et	H	Me	H	Me	4,8,10,12-Tetradesmethyl-10,12-diethylerythromycin A
Et	Et	H	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A
Et	Et	H	H	Me	Me	6,8,10,12-Tetradesmethyl-10,12-diethylerythromycin A
Et	H	Et	Me	Me	Et	2,8,10,12-Tetradesmethyl-2,8,12-triethylerythromycin A
Et	H	Et	Me	Me	H	2,8,10,12-Tetradesmethyl-8,12-diethylerythromycin A
Et	H	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8,12-triethylerythromycin A
Et	H	Et	Me	H	Me	4,8,10,12-Tetradesmethyl-8,12-diethylerythromycin A
Et	H	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8,12-triethylerythromycin A
Et	H	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8,12-diethylerythromycin A
Et	Et	Et	Me	Me	Et	2,6,10,12-Tetradesmethyl-2,8,10,12-tetraethylerythromycin A
Et	Et	Et	Me	Me	H	2,6,10,12-Tetradesmethyl-8,10,12-triethylerythromycin A
Et	Et	Et	Me	Et	Me	4,8,10,12-Tetradesmethyl-4,8,10,12-tetraethylerythromycin A
Et	Et	Et	Mc	H	Me	4,8,10,12-Tetradesmethyl-8,10,12-triethylerythromycin
Et	Et	Et	Et	Me	Me	6,8,10,12-Tetradesmethyl-6,8,10,12-tetraethylerythromycin A
Et	Et	Et	H	Me	Me	6,8,10,12-Tetradesmethyl-8,10,12-triethylerythromycin
H	H	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2-ethylerythromycin A
H	H	Me	H	Mc	H	2,6,10,12-Tetradesmethylerythromycin A
H	H	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4- ethylerythromycin A
H	H	Me	H	H	Me	4,6,10,12-Tetradesmethylerythromycin A
H	Et	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2,10-diethylerythromycin A
H	Et	Me	H	Me	H	2,6,10,12-Tetradesmethyl-10-ethylerythromycin A
H	Et	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4,10-diethylerythromycin A

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H	Et	Me	H	H	Me	4,6,10,12-Tetradesmethyl-10-ethylerythromycin A
H	H	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6-diethylerythromycin A
H	H	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6-ethylerythromycin A
H	H	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6-diethylerythromycin A
H	H	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6-ethylerythromycin A
H	Et	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,10-triethylerythromycin A
H	Et	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6,10-diethylerythromycin A
H	Et	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6,10-triethylerythromycin A
H	Et	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,10-diethylerythromycin A
Et	H	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2,12-diethylerythromycin A
Et	H	Me	H	Me	H	2,6,10,12-Tetradesmethyl-12-ethylerythromycin A
Et	H	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4,12-diethylerythromycin A
Et	H	Me	H	H	Me	4,6,10,12-Tetradesmethyl-12-ethylerythromycin A
Et	Et	Me	H	Me	Et	2,6,10,12-Tetradesmethyl-2,10,12-triethylerythromycin A
Et	Et	Me	H	Me	H	2,6,10,12-Tetradesmethyl-10,12-diethylerythromycin A
Et	Et	Me	H	Et	Me	4,6,10,12-Tetradesmethyl-4,10,12-triethylerythromycin A
Et	Et	Me	H	H	Me	4,6,10,12-Tetradesmethyl-10,12-diethylerythromycin A
Et	H	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,12-triethylerythromycin A
Et	H	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6,12--diethylerythromycin A
Et	H	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6,12-triethylerythromycin A
Et	H	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,12--diethylerythromycin A
Et	Et	Me	Et	Me	Et	2,6,10,12-Tetradesmethyl-2,6,10,12-tetraethylerythromycin A
Et	Et	Me	Et	Me	H	2,6,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A
Et	Et	Me	Et	Et	Me	4,6,10,12-Tetradesmethyl-4,6,10,12-tetraethylerythromycin A
Et	Et	Me	Et	H	Me	4,6,10,12-Tetradesmethyl-6,10,12-triethylerythromycin A
H	H	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2-ethylerythromycin A
H	H	Me	Me	H	H	2,4,10,12-Tetradesmethylerythromycin A
H	Et	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2,10-diethylerythromycin A
H	Et	Me	Me	H	H	2,4,10,12-Tetradesmethyl-10-ethylerythromycin A
H	H	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4-diethylerythromycin A
H	H	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4-ethylerythromycin A
H	Et	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4,10-triethylerythromycin A
H	Et	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4,10-diethylerythromycin A
Et	H	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2,12-diethylerythromycin A
Et	H	Me	Me	H	H	2,4,10,12-Tetradesmethyl-12-ethylerythromycin A
Et	Et	Me	Me	H	Et	2,4,10,12-Tetradesmethyl-2,10,12-triethylerythromycin A
Et	Et	Me	Me	H	H	2,4,10,12-Tetradesmethyl-10,12-diethylerythromycin A
Et	H	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4,12-triethylerythromycin A
Et	H	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4,12-diethylerythromycin A
Et	Et	Me	Me	Et	Et	2,4,10,12-Tetradesmethyl-2,4,10,12-tetraethylerythrpmycin A
Et	Et	Me	Me	Et	H	2,4,10,12-Tetradesmethyl-4,10,12-triethylerythromycin A
H	Me	H	H	Me	Et	2,6,8,12-Tetradesmethyl-2-ethylerythromycin A
H	Me	H	H	Me	H	2,6,8,12-Tetradesmethylerythromycin A
H	Me	H	H	Et	Me	4,6,8,12-Tetradesmethyl-4--ethylerythromycin A
H	Me	H	H	H	Me	4,6,8,12-Tetradesmethylerythromycin A
H	Me	Et	H	Me	Et	2,6,8,12-Tetradesmethyl-2,8-diethylerythromycin A
H	Me	Et	H	Me	H	2,6,8,12-Tetradesmethyl-8--ethylerythromycin A
H	Me	Et	H	Et	Me	4,6,8,12-Tetradesmethyl-4,,8-diethylerythromycin A
H	Me	Et	H	H	Me	4,6,8,12-Tetradesmethyl-8-ethylerythromycin A
H	Me	H	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6-diethylerythromycin A
H	Me	H	Et	Me	H	2,6,8,12-Tetradesmethyl-6-ethylerythromycin A
H	Me	H	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6-diethylerythromycin A
H	Me	H	Et	H	Me	4,6,8,12-Tetradesmethyl-6-ethylerythromycin A
H	Me	Et	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6,8-triethylerythromycin A

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H	Me	Et	Et	Me	H	2,6,8,12-Tetradesmethyl-6,8-diethylerythromycin A
H	Me	Et	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6,8-triethylerythromycin A
H	Me	Et	Et	H	Me	4,6,8,12-Tetradesmethyl-6,8-diethylerythromycin A
Et	Me	H	H	Me	Et	2,6,8,12-Tetradesmethyl-2,12-diethylerythromycin A
Et	Me	H	H	Me	H	2,6,8,12-Tetradesmethyl-12-ethylerythromycin A
Et	Me	H	H	Et	Me	4,6,8,12-Tetradesmethyl-4,12-diethylerythromycin A
Et	Me	H	H	H	Me	4,6,8,12-Tetradesmethyl-12-ethylerythromycin A
Et	Me	Et	H	Me	Et	2,6,8,12-Tetradesmethyl-2,8,12-triethylerythromycin A
Et	Me	Et	H	Me	H	2,6,8,12-Tetradesmethyl-8,12-diethylerythromycin A
Et	Me	Et	H	Et	Me	4,6,8,12-Tetradesmethyl-4,8,12-triethylerythromycin A
Et	Me	Et	H	H	Me	4,6,8,12-Tetradesmethyl-8,12-diethylerythromycin A
Et	Me	H	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6,12-triethylerythromycin A
Et	Me	H	Et	Me	H	2,6,8,12-Tetradesmethyl-6,12-diethylerythromycin A
Et	Me	H	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6,12-triethylerythromycin A
Et	Me	H	Et	H	Me	4,6,8,12-Tetradesmethyl-6,12-diethylerythromycin A
Et	Me	Et	Et	Me	Et	2,6,8,12-Tetradesmethyl-2,6,8,12-tetraethylerythromycin A
Et	Me	Et	Et	Me	H	2,6,8,12-Tetradesmethyl-6,8,12-triethylerythromycin A
Et	Me	Et	Et	Et	Me	4,6,8,12-Tetradesmethyl-4,6,8,12-tetraethylerythromycin A
Et	Me	Et	Et	H	Me	4,6,8,12-Tetradesmethyl-6,8,12-triethylerythromycin A
H	Me	Me	H	H	Et	2,4,6,12-Tetradesmethyl-2-ethylerythromycin A
H	Me	Me	H	H	H	2,4,6,12-Tetradesmethylerythromycin A
H	Me	Me	Et	H	Et	2,4,6,12-Tetradesmethyl-2,6-diethylerythromycin A
H	Me	Me	Et	H	H	2,4,6,12-Tetradesmethyl-6-ethylerythromycin A
H	Me	Me	H	Et	Et	2,4,6,12-Tetradesmethyl-2,4-diethylerythromycin A
H	Me	Me	H	Et	H	2,4,6,12-Tetradesmethyl-4-ethylerythromycin A
H	Me	Me	Et	Et	Et	2,4,6,12-Tetradesmethyl-2,4,6-triethylerythromycin A
H	Me	Me	Et	Et	H	2,4,6,12-Tetradesmethyl-4,6-diethylerythromycin A
Et	Me	Me	H	H	Et	2,4,6,12-Tetradesmethyl-2,12-diethylerythromycin A
Et	Me	Me	H	H	H	2,4,6,12-Tetradesmethyl-12-ethylerythromycin A
Et	Me	Me	Et	H	Et	2,4,6,12-Tetradesmethyl-2,6,12-triethylerythromycin A
Et	Me	Me	Et	H	H	2,4,6,12-Tetradesmethyl-6,12-diethylerythromycin A
Et	Me	Me	H	Et	Et	2,4,6,12-Tetradesmethyl-2,4,12-triethylerythromycin A
Et	Me	Me	H	Et	H	2,4,6,12-Tetradesmethyl--diethylerythromycin A
Et	Me	Me	Et	Et	Et	2,4,6,12-Tetradesmethyl-2,4,6,12-tetraethylerythromycin A
Et	Me	Me	Et	Et	H	2,4,6,12-Tetradesmethyl-4,6,12-triethylerythromycin A
Me	H	H	H	Me	Et	2,6,8,10-Tetradesmethyl-2-ethylerythromycin A
Me	H	H	H	Me	H	2,6,8,10-Tetradesmethylerythromycin A
Me	H	H	H	Et	Me	4,6,8,10-Tetradesmethyl-4-ethylerythromycin A
Me	H	H	H	H	Me	4,6,8,10-Tetradesmethylerythromycin A
Me	H	Et	H	Me	Et	2,6,8,10-Tetradesmethyl-2,8-diethylerythromycin A
Me	H	Et	H	Me	H	2,6,8,10-Tetradesmethyl-8-ethylerythromycin A
Me	H	Et	H	Et	Me	4,6,8,10-Tetradesmethyl-4,8-diethylerythromycin A
Me	H	Et	H	H	Me	4,6,8,10-Tetradesmethyl-8-ethylerythromycin A
Me	H	H	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6-diethylerythromycin A
Me	H	H	Et	Me	H	2,6,8,10-Tetradesmethyl-6-ethylerythromycin A
Me	H	H	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6-diethylerythromycin A
Me	H	H	Et	H	Me	4,6,8,10-Tetradesmethyl-6-ethylerythromycin A
Me	H	Et	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6,8-triethylerythromycin A
Me	H	Et	Et	Me	H	2,6,8,10-Tetradesmethyl-6,8-diethylerythromycin A
Me	H	Et	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6,8-triethylerythromycin A
Me	H	Et	Et	H	Me	4,6,8,10-Tetradesmethyl-6,8-diethylerythromycin A
Me	Et	H	H	Me	Et	2,6,8,10-Tetradesmethyl-2,10-diethylerythromycin A
Me	Et	H	H	Me	H	2,6,8,10-Tetradesmethyl-10-ethylerythromycin A
Me	Et	H	H	Et	Me	4,6,8,10-Tetradesmethyl-4,10-diethylerythromycin A

Me	Et	H	H	H	Me	4,6,8,10-Tetradesmethyl-10-ethylerythromycin A
Me	Et	Et	H	Me	Et	2,6,8,10-Tetradesmethyl-2,8,10-triethylerythromycin A
Me	Et	Et	H	Me	H	2,6,8,10-Tetradesmethyl-8,10-diethylerythromycin A
Me	Et	Et	H	Et	Me	4,6,8,10-Tetradesmethyl-4,8,10-triethylerythromycin A
Me	Et	Et	H	H	Me	4,6,8,10-Tetradesmethyl-8,10-diethylerythromycin A
Me	Et	H	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6,10-triethylerythromycin A
Mc	Et	H	Et	Me	H	2,6,8,10-Tetradesmethyl-6,10-diethylerythromycin A
Me	Et	H	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6,10-triethylerythromycin A
Me	Et	H	Et	H	Me	4,6,8,10-Tetradesmethyl-6,10-diethylerythromycin A
Me	Et	Et	Et	Me	Et	2,6,8,10-Tetradesmethyl-2,6,8,10-tetraethylerythromycin A
Me	Et	Et	Et	Me	H	2,6,8,10-Tetradesmethyl-6,8,10-triethylerythromycin A
Mc	Et	Et	Et	Et	Me	4,6,8,10-Tetradesmethyl-4,6,8,10-tetraethylerythromycin A
Me	Et	Et	Et	H	Mc	4,6,8,10-Tetradesmethyl-6,8,10-triethylerythromycin A
Me	H	H	Me	H	Et	2,4,8,10-Tetradesmethyl-2-ethylerythromycin A
Me	H	H	Me	H	H	2,4,8,10-Tetradesmethylerythromycin A
Me	H	Et	Me	H	Et	2,4,8,10-Tetradesmethyl-2,8-diethylerythromycin A
Me	H	Et	Me	H	H	2,4,8,10-Tetradesmethyl-8-ethylerythromycin A
Mc	H	H	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4-diethylerythromycin A
Me	H	H	Me	Et	H	2,4,8,10-Tetradesmethyl-4-ethylerythromycin A
Me	H	Et	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4,8-triethylerythromycin A
Me	H	Et	Me	Et	H	2,4,8,10-Tetradesmethyl-4,8-diethylerythromycin A
Me	Et	H	Mc	H	Et	2,4,8,10-Tetradesmethyl-2,10-diethylerythromycin A
Me	Et	H	Me	H	H	2,4,8,10-Tetradesmethyl-10-ethylerythromycin A
Me	Et	Et	Me	H	Et	2,4,8,10-Tetradesmethyl-2,8,10-triethylerythromycin A
Me	Et	Et	Me	H	H	2,4,8,10-Tetradesmethyl-8,10-diethylerythromycin A
Me	Et	H	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4,10-triethylerythromycin A
Me	Et	H	Me	Et	H	2,4,8,10-Tetradesmethyl-4,10-diethylerythromycin A
Me	Et	Et	Me	Et	Et	2,4,8,10-Tetradesmethyl-2,4,8,10-tetraethylerythromycin A
Me	Et	Et	Me	Et	H	2,4,8,10-Tetradesmethyl-4,8,10-triethylerythromycin A
Me	Me	H	H	H	Et	2,4,6,8-Tetradesmethyl-2-ethylerythromycin A
Me	Me	H	H	H	H	2,4,6,8-Tetradesmethylerythromycin A
Me	Mc	H	Et	H	Et	2,4,6,8-Tetradesmethyl-2,6,-diethylerythromycin A
Me	Mc	H	Et	H	H	2,4,6,8-Tetradesmethyl-6-ethylerythromycin A
Me	Me	H	H	Et	Et	2,4,6,8-Tetradesmethyl-2,4-diethylerythromycin A
Me	Me	H	H	Et	H	2,4,6,8-Tetradesmethyl-4-ethylerythromycin A
Me	Me	H	Et	Et	Et	2,4,6,8-Tetradesmethyl-2,4,6-triethylerythromycin A
Me	Me	H	Et	Et	H	2,4,6,8-Tetradesmethyl-4,6-diethylerythromycin A
Me	Me	Et	H	H	Et	2,4,6,8-Tetradesmethyl-2,8-diethylerythromycin A
Me	Me	Et	H	H	H	2,4,6,8-Tetradesmethyl-8-ethylerythromycin A
Me	Me	Et	Et	H	Et	2,4,6,8-Tetradesmethyl-2,6,8-triethylerythromycin A
Me	Me	Et	Et	H	H	2,4,6,8-Tetradesmethyl-6,8-diethylerythromycin A
Me	Me	Et	H	Et	Et	2,4,6,8-Tetradesmethyl-2,4,8-triethylerythromycin A
Me	Me	Et	H	Et	H	2,4,6,8-Tetradesmethyl-4,8-diethylerythromycin A
Me	Me	Et	Et	Et	Et	2,4,6,8-Tetradesmethyl-2,4,6,8-tetraethylerythromycin A
Me	Me	Et	Et	Et	H	2,4,6,8-Tetradesmethyl-4,6,8-triethylerythromycin A

E. Five Changes

H	H	H	H	H	Me	4,6,8,10,12-Pentadesmethylerythromycin A
Et	H	H	H	H	Me	4,6,8,10,12-Pentadesmethyl-12-ethylerythromycin A
H	Et	H	H	H	Me	4,6,8,10,12-Pentadesmethyl-10-ethylerythromycin A
H	H	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8-ethylerythromycin A
H	H	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6-ethylerythromycin A
H	H	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4-ethylerythromycin A
Et	Et	H	H	H	Me	4,6,8,10,12-Pentadesmethyl-10,12-diethylerythromycin A

Et	H	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8,12-diethylerythromycin A
Et	H	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,12-diethylerythromycin A
Et	H	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,12-diethylerythromycin A
H	Et	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8,10-diethylerythromycin A
H	Et	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,10-diethylerythromycin A
H	Et	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,10-diethylerythromycin A
H	H	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8-diethylerythromycin A
H	H	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8-diethylerythromycin A
H	H	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6-diethylerythromycin A
Et	Et	Et	H	H	Me	4,6,8,10,12-Pentadesmethyl-8,10,12-triethylerythromycin A
Et	Et	H	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,10,12-triethylerythromycin A
Et	Et	H	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,10,12-triethylerythromycin A
Et	H	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8,12-triethylerythromycin A
Et	H	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8,12-triethylerythromycin A
Et	H	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,12-triethylerythromycin A
H	Et	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8,10-triethylerythromycin A
H	Et	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8,10-triethylerythromycin A
H	Et	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,10-triethylerythromycin A
H	H	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8-triethylerythromycin A
Et	Et	Et	Et	H	Me	4,6,8,10,12-Pentadesmethyl-6,8,10,12-tetraethylerythromycin A
Et	Et	Et	H	Et	Me	4,6,8,10,12-Pentadesmethyl-4,8,10,12-tetraethylerythromycin A
Et	Et	H	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,10,12-tetraethylerythromycin A
Et	H	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8,12-tetraethylerythromycin A
H	Et	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8,10-tetraethylerythromycin A
Et	Et	Et	Et	Et	Me	4,6,8,10,12-Pentadesmethyl-4,6,8,10,12-pentaethylerythromycin A
H	H	H	H	Me	H	2,6,8,10,12-Pentadesmethylerythromycin A
Et	H	H	H	Me	H	2,6,8,10,12-Pentadesmethyl-12-ethylerythromycin A
H	Et	H	H	Me	H	2,6,8,10,12-Pentadesmethyl-10-ethylerythromycin A
H	H	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8-ethylerythromycin A
H	H	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6-ethylerythromycin A
H	H	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2-ethylerythromycin A
Et	Et	H	H	Me	H	2,6,8,10,12-Pentadesmethyl-10,12-diethylerythromycin A
Et	H	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8,12-diethylerythromycin A
Et	H	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-4,12-diethylerythromycin A
Et	H	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,12-diethylerythromycin A
H	Et	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8,10-diethylerythromycin A
H	Et	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,10-diethylerythromycin A
H	Et	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,10-diethylerythromycin A
H	H	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8-diethylerythromycin A
H	H	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8-diethylerythromycin A
H	H	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6-diethylerythromycin A
Et	Et	Et	H	Me	H	2,6,8,10,12-Pentadesmethyl-8,10,12-triethylerythromycin A
Et	Et	H	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,10,12-triethylerythromycin A
Et	Et	H	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,10,12-triethylerythromycin A
Et	H	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8,12-triethylerythromycin A
Et	H	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8,12-triethylerythromycin A
Et	H	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,12-triethylerythromycin A
H	Et	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8,10-triethylerythromycin A
H	Et	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8,10-triethylerythromycin A
H	Et	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,10-triethylerythromycin A
H	H	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,8-triethylerythromycin A
Et	Et	Et	Et	Me	H	2,6,8,10,12-Pentadesmethyl-6,8,10,12-tetraethylerythromycin A
Et	Et	Et	H	Me	Et	2,6,8,10,12-Pentadesmethyl-2,8,10,12-tetraethylerythromycin A

Et	Et	H	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,10,12-tetraethylerythromycin
AEt	H	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,,8,12-tetraethylerythromycin A
H	Et	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,8,10-tetraethylerythromycin A
Et	Et	Et	Et	Me	Et	2,6,8,10,12-Pentadesmethyl-2,6,,8,10,12-pentaethylerythromycin A
H	H	H	Me	H	H	2,4,8,10,12-Pentadesmethylerythromycin A
Et	H	H	Me	H	H	2,4,8,10,12-Pentadesmethyl-12-ethylerythromycin A
H	Et	H	Me	H	H	2,4,8,10,12-Pentadesmethyl-10-ethylerythromycin A
H	H	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8-ethylerythromycin A
H	H	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4-ethylerythromycin A
H	H	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2-ethylerythromycin A
Et	Et	H	Me	H	H	2,4,8,10,12-Pentadesmethyl-10,12-diethylerythromycin A
Et	H	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8,12-diethylerythromycin A
Et	H	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,12-diethylerythromycin A
Et	H	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,12-diethylerythromycin A
H	Et	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8,10-diethylerythromycin A
H	Et	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,10-diethylerythromycin A
H	Et	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,10-diethylerythromycin A
H	H	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8-diethylerythromycin A
H	H	Et	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,8-diethylerythromycin A
H	H	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4-diethylerythromycin A
Et	Et	Et	Me	H	H	2,4,8,10,12-Pentadesmethyl-8,10,12-triethylerythromycin A
Et	Et	H	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,10,12-triethylerythromycin A
Et	Et	H	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,10,12-triethylerythromycin A
Et	H	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8,12-triethylerythromycin A
Et	H	Et	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,8,12-triethylerythromycin A
Et	H	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,12-triethylerythromycin A
H	Et	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8,10-triethylerythromycin A
H	Et	Et	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,8,10-triethylerythromycin A
H	Et	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,10-triethylerythromycin A
H	H	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8-triethylerythromycin A
Et	Et	Et	Me	Et	H	2,4,8,10,12-Pentadesmethyl-4,8,10,12-tetraethylerythromycin A
Et	Et	Et	Me	H	Et	2,4,8,10,12-Pentadesmethyl-2,8,10,12-tetraethylerythromycin A
Et	Et	H	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,10,12-tetraethylerythromycin A
Et	H	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8,12-tetraethylerythromycin A
H	Et	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8,10-tetraethylerythromycin A
Et	Et	Et	Me	Et	Et	2,4,8,10,12-Pentadesmethyl-2,4,8,10,12-pentaethylerytthromycin A
H	H	Me	H	H	H	2,4,6,10,12-Pentadesmethylerythromycin
Et	H	Me	H	H	H	2,4,6,10,12-Pentadesmethyl-12-ethylerythromycin A
H	Et	Me	H	H	H	2,4,6,10,12-Pentadesmethyl-10-ethylerythromycin A
H	H	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6-ethylerythromycin A
H	H	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4-ethylerythromycin A
H	H	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2-ethylerythromycin A
Et	Et	Me	H	H	H	2,4,6,10,12-Pentadesmethyl-10,12-diethylerythromycin A
Et	H	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6,12-diethylerythromycin A
Et	H	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4,12-diethylerythromycin A
Et	H	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2,12-diethylerythromycin A
H	Et	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6,10-diethylerythromycin A
H	Et	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4,10-diethylerythromycin A
H	Et	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2,10-diethylerythromycin A
H	H	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6-diethylerythromycin A
H	H	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6-diethylerythromycin A
H	H	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4-diethylerythromycin A
Et	Et	Me	Et	H	H	2,4,6,10,12-Pentadesmethyl-6,10,12-triethylerythromycin A
Et	Et	Me	H	Et	H	2,4,6,10,12-Pentadesmethyl-4,10,12-triethylerythromycin A

Et	Et	Me	H	H	Et	2,4,6,10,12-Pentadesmethyl-2,10,12-triethylerythromycin A
Et	H	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6,12-triethylerythromycin A
Et	H	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6,12-triethylerythromycin A
Et	H	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,12-triethylerythromycin A
H	Et	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6,10-triethylerythromycin A
H	Et	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6,10-triethylerythromycin A
H	Et	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,10-triethylerythromycin A
H	H	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6-triethylerythromycin A
Et	Et	Me	Et	Et	H	2,4,6,10,12-Pentadesmethyl-4,6,10,12-tetraethylerythromycin A
Et	Et	Me	Et	H	Et	2,4,6,10,12-Pentadesmethyl-2,6,10,12-tetraethylerythromycin A
Et	Et	Me	H	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,10,12-tetraethylerythromycin A
Et	H	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6,12-tetraethylerythromycin A
H	Et	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6,10-tetraethylerythromycin A
Et	Et	Me	Et	Et	Et	2,4,6,10,12-Pentadesmethyl-2,4,6,10,12-pentaethylerythromycin A
H	Me	H	H	H	H	2,4,6,8,12-Pentadesmethylerythromycin A
Et	Me	H	H	H	H	2,4,6,8,12-Pentadesmethyl-12-ethylerythromycin A
H	Me	Et	H	H	H	2,4,6,8,12-Pentadesmethyl-8-ethylerythromycin A
H	Me	H	Et	H	H	2,4,6,8,12-Pentadesmethyl-6-ethylerythromycin A
H	Me	H	H	Et	H	2,4,6,8,12-Pentadesmethyl-4-ethylerythromycin A
H	Me	H	H	H	Et	2,4,6,8,12-Pentadesmethyl-2-ethylerythromycin A
Et	Me	Et	H	H	H	2,4,6,8,12-Pentadesmethyl-8,12-diethylerythromycin A
Et	Me	H	Et	H	H	2,4,6,8,12-Pentadesmethyl-6,12-diethylerythromycin A
Et	Me	H	H	Et	H	2,4,6,8,12-Pentadesmethyl-4,12-diethylerythromycin A
Et	Me	H	H	H	Et	2,4,6,8,12-Pentadesmethyl-2,12-diethylerythromycin A
H	Me	Et	Et	H	H	2,4,6,8,12-Pentadesmethyl-6,8-diethylerythromycin A
H	Me	Et	H	Et	H	2,4,6,8,12-Pentadesmethyl-4,8-diethylerythromycin A
H	Me	Et	H	H	Et	2,4,6,8,12-Pentadesmethyl-2,8-diethylerythromycin A
H	Me	H	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6-diethylerythromycin A
H	Me	H	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6-diethylerythromycin A
H	Me	H	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4-diethylerythromycin A
Et	Me	Et	Et	H	H	2,4,6,8,12-Pentadesmethyl-6,8,12-triethylerythromycin A
Et	Me	Et	H	Et	H	2,4,6,8,12-Pentadesmethyl-4,8,12-triethylerythromycin A
Et	Me	Et	H	H	Et	2,4,6,8,12-Pentadesmethyl-2,8,12-triethylerythromycin A
Et	Me	H	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6,12-triethylerythromycin A
Et	Me	H	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6,12-triethylerythromycin A
Et	Me	H	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,12-triethylerythromycin A
H	Me	Et	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6,8-triethylerythromycin A
H	Me	Et	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6,8-triethylerythromycin A
H	Me	Et	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,8-triethylerythromycin A
H	Me	H	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6-triethylerythromycin A
Et	Me	Et	Et	Et	H	2,4,6,8,12-Pentadesmethyl-4,6,8-triethylerythromycin A
Et	Me	Et	Et	H	Et	2,4,6,8,12-Pentadesmethyl-2,6,8,12-tetraethylerythromycin A
Et	Me	Et	H	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,8,12-tetraethylerythromycin A
Et	Me	H	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6,12-tetraethylerythromycin A
H	Me	Et	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6,8-tetraethylerythromycin A
Et	Me	Et	Et	Et	Et	2,4,6,8,12-Pentadesmethyl-2,4,6,8,12-pentaethylerythromycin A
Me	H	H	H	H	H	2,4,6,8,10-Pentadesmethylerythromycin A
Me	Et	H	H	H	H	2,4,6,8,10-Pentadesmethyl-10-ethylerythromycin A
Me	H	Et	H	H	H	2,4,6,8,10-Pentadesmethyl-8-ethylerythromycin A
Me	H	H	Et	H	H	2,4,6,8,10-Pentadesmethyl-6-ethylerythromycin A
Me	H	H	H	Et	H	2,4,6,8,10-Pentadesmethyl-4-ethylerythromycin A
Me	H	H	H	H	Et	2,4,6,8,10-Pentadesmethyl-2-ethylerythromycin A
Me	Et	Et	H	H	H	2,4,6,8,10-Pentadesmethyl-8,10 diethylerythromycin A
Me	Et	H	Et	H	H	2,4,6,8,10-Pentadesmethyl-6,10 diethylerythromycin A

(Line numbers in left margin: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50)

Me	Et	H	H	Et	H	2,4,6,8,10-Pentadesmethyl-4,10 diethylerythromycin A
Me	Et	H	H	H	Et	2,4,6,8,10-Pentadesmethyl-2,10 diethylerythromycin A
Me	H	Et	Et	H	H	2,4,6,8,10-Pentadesmethyl-6,8-diethylerythromycin A
Me	H	Et	H	Et	H	2,4,6,8,10-Pentadesmethyl-4,8-diethylerythromycin A
Me	H	Et	H	H	Et	2,4,6,8,10-Pentadesmethyl-2,8-diethylerythromycin A
Me	H	H	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6-diethylerythromycin A
Me	H	H	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6-diethylerythromycin A
Me	H	H	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4-diethylerythromycin A
Me	Et	Et	Et	H	H	2,4,6,8,10-Pentadesmethyl-6,8,10-triethylerythromycin A
Me	Et	Et	H	Et	H	2,4,6,8,10-Pentadesmethyl-4,8,10-triethylerythromycin A
Me	Et	Et	H	H	Et	2,4,6,8,10-Pentadesmethyl-2,8,10-triethylerythromycin A
Me	Et	H	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6,10-triethylerythromycin A
Me	Et	H	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6,10-triethylerythromycin A
Me	Et	H	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,10-triethylerythromycin A
Me	H	Et	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6,8-triethylerythromycin A
Me	H	Et	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6,8-triethylerythromycin A
Me	H	Et	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,8-triethylerythromycin A
Me	H	H	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6-triethylerythromycin A
Me	Et	Et	Et	Et	H	2,4,6,8,10-Pentadesmethyl-4,6,8,10-tetraethylerythromycin A
Me	Et	Et	Et	H	Et	2,4,6,8,10-Pentadesmethyl-2,6,8,10-tetraethylerythromycin A
Me	Et	Et	H	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,8,10-tetraethylerythromycin A
Me	Et	H	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6,10-tetraethylerythromycin A
Me	H	Et	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6,8-tetraethylerythromycin A
Me	Et	Et	Et	Et	Et	2,4,6,8,10-Pentadesmethyl-2,4,6,8,10-pentaethylerythromycin A

F. Six Changes

H	H	H	H	H	H	2,4,6,8,10,12-Hexadesmethylerythromycin A
Et	H	H	H	H	H	2,4,6,8,10,12-Hexadesmethyl-12-ethylerythromycin A
H	Et	H	H	H	H	2,4,6,8,10,12-Hexadesmethyl-10-ethylerythromycin A
H	H	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8-ethylerythromycin A
H	H	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6-ethylerythromycin A
H	H	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4-ethylerythromycin A
H	H	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2-ethylerythromycin A
Et	Et	H	H	H	H	2,4,6,8,10,12-Hexadesmethyl-10,12-diethylerythromycin A
Et	H	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8,12-diethylerythromycin A
Et	H	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,12-diethylerythromycin A
Et	H	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,12-diethylerythromycin A
Et	H	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,12-diethylerythromycin A
H	Et	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8,10-diethylerythromycin A
H	Et	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,10-diethylerythromycin A
H	Et	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,10-diethylerythromycin A
H	Et	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,10-diethylerythromycin A
H	H	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8-diethylerythromycin A
H	H	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8-diethylerythromycin A
H	H	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8-diethylerythromycin A
H	H	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6-diethylerythromycin A
H	H	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6-diethylerythromycin A
H	H	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4-diethylerythromycin A
Et	Et	Et	H	H	H	2,4,6,8,10,12-Hexadesmethyl-8,10,12-triethylerythromycin A
Et	Et	H	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,10,12-triethylerythromycin A
Et	Et	H	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,10,12-triethylerythromycin A
Et	Et	H	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,10,12-triethylerythromycin A
Et	H	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8,12-triethylerythromycin A
Et	H	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8,12-triethylerythromycin A

Et	H	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8,12-triethylerythromycin A
Et	H	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,12-triethylerythromycin A
Et	H	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,12-triethylerythromycin A
Et	H	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,12-triethylerythromycin A
H	Et	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8,10-triethylerythromycin A
H	Et	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8,10-triethylerythromycin A
H	Et	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8,10-triethylerythromycin A
H	Et	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,10-triethylerythromycin A
H	Et	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,10-triethylerythromycin A
H	Et	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,10-triethylerythromycin A
H	H	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8-triethylerythromycin A
H	H	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8-triethylerythromycin A
H	H	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8-triethylerythromycin A
H	H	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6-triethylerythromycin A
Et	Et	Et	Et	H	H	2,4,6,8,10,12-Hexadesmethyl-6,8,10,12-tetraethylerythromycin A
Et	Et	Et	H	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,8,10,12-tetraethylerythromycin A
Et	Et	Et	H	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,8,10,12-tetraethylerythromycin A
Et	Et	H	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,10,12-tetraethylerythromycin A
Et	Et	H	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,10,12-tetraethylerythromycin A
Et	Et	H	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,10,12-tetraethylerythromycin A
Et	H	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8,12-tetraethylerythromycin A
Et	H	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8,12-tetraethylerythromycin A
Et	H	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8,12-tetraethylerythromycin A
Et	H	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,12-tetraethylerythromycin A
H	Et	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8,10-tetraethylerythromycin A
H	Et	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8,10-tetraethylerythromycin A
H	Et	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8,10-tetraethylerythromycin A
H	Et	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,10-tetraethylerythromycin A
H	H	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8-tetraethylerythromycin A
Et	Et	Et	Et	Et	H	2,4,6,8,10,12-Hexadesmethyl-4,6,8,10,12-pentaethylerythromycin A
Et	Et	Et	Et	H	Et	2,4,6,8,10,12-Hexadesmethyl-2,6,8,10,12-pentaethylerythromycin A
Et	Et	Et	H	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,8,10,12-pentaethylerythromycin A
Et	Et	H	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,10,12-pentaethylerythromycin A
Et	H	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,,8,12-pentaethylerythromycin A
H	Et	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8,10-pentaethylerythromycin A
Et	Et	Et	Et	Et	Et	2,4,6,8,10,12-Hexadesmethyl-2,4,6,8,10,12-hexaethylerythromycin

Although in the Examples that follow the AT-encoding DNA fragments from *S. hygroscopicus* ATCC 29253, *S. venezuelae* ATCC 15439, and *S. caelestis* NRRL-2821 were used to replace resident AT-encoding DNA fragments in the eryPKS to yield desmethyl, desmethylethyl, and desmethylhydroxyerythromycins, it is understood that many malonate, ethylmalonate, and hydroxymalonate AT-encoding DNA fragments can be used in place of or in addition to the heterologous malonate, ethylmalonate, and hydroxymalonate-AT DNA fragments described herein to produce the same desmethyl, desmethylethyl, and desmethylhydroxyerythromycin compounds. Examples of DNA fragments encoding malonate-AT domains that can be used in place of or in addition to those specifically described in the Examples below include but are not limited to the DNA fragments encoding AT domains from modules 2, 5, 8, 9, 11, or 12 of the rapamycin PKS genes from *S.*

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hygroscopicus, the AT domain from module 2 of the PKS responsible the synthesis of methymycin or pikromycin by *S. venezuelae,* the AT domains from modules 3 and 7 of the PKS responsible for the synthesis of tylosin by *S. fradiae,* or the AT domains from modules 1, 2, 3 and 7 of the PKS responsible for the synthesis of spiramycin by *S. ambofaciens.*

5 Examples of DNA fragments encoding ethylmalonate-AT domains that can be used in place of or in addition to those specifically described in the Examples below include but are not limited to the DNA fragments encoding the AT domain from module 5 of the spiramycin PKS genes from *S. ambofaciens,* the AT domain from module 5 of the tylosin PKS genes from *S. fradiae,* and the AT domain from module 5 of the maridomycin PKS genes of *S.*

10 *hygroscopicus.* Examples of DNA fragments encoding hydroxymalonate-AT domains that can be used in place of or in addition to those specifically described in the Examples below include but are not limited to the DNA fragments encoding the AT domain from module 6 of the spiramycin PKS genes from *S. ambofaciens,* the AT domain from module 6 of the maridomycin PKS genes from *S. hygroscopicus,* and the AT domain from module 6 of the

15 leucomycin PKS genes from *Streptoverticillium kitasatoensis.* Thus the use of any and all DNA fragments encoding malonate, ethylmalonate, and hydroxymalonate-ATs to replace any of the resident DNA fragments encoding methylmalonate-ATs in the eryPKS genes to result in the production of novel derivatives of erythromycin are considered within the scope of the present invention.

20 Furthermore, whereas the NidAT6 domain was exemplified herein to replace the AT domains of the starter, module 1 or module 2 in the eryPKS to introduce hydroxyl groups into positions 14, 12 and 10, respectively of the polyketide backbone of erythromycin, it is understood that the NidAT6 domain can also be used to replace the AT domains of modules 3, 4, 5 or 6 of the eryPKS to result in the production of erythromycin derivatives containing a

25 hydroxyl group at position 8, 6, 4 or 2, respectively, of the erythromycin backbone to replace the methyl group that is normally seen at the corresponding position. Therefore, all compounds produced from the replacement of an eryAT domain with the NidAT6, including the compounds 8-desmethyl-8-hydroxyerythromycin A, 6-desmethyl-6-epierythromycin A, 4-desmethyl-4-hydroxyerythromycin A and 2-desmethyl-2-hydroxyerythromycin A or their

30 6-deoxy derivatives and the corresponding strains that produce them are included under the scope of this invention.

Furthermore, whereas the NidAT6 domain was exemplified herein to replace a single AT domain in the eryPKS to produce a derivatized erythromycin A molecule containing a single additional hydroxyl group, those skilled in the art understand that it is possible to

35 independently replace two or more AT domains of the eryPKS with the NidAT6 domain to obtain derivatized erythromycins with two or more additional hydroxyl groups. Examples of

erythromycin molecules containing two additional hydroxyl groups include, but are not
limited to, 2,12-didesmethyl-2,12-dihydroxyerythromycin, 4,10-didesmethyl-4,10-
dihydroxyerythromycin, and the like. Therefore, all compounds produced from the
replacement of two or more AT domains of the eryPKS with NidAT6 and the corresponding

5   strains that make them are included under the scope of this invention.

It is also understood by those skilled in the art that the placement of the NidAT6
domain at more than a single position in the eryPKS may result in the genetic instability of
the hybrid PKS DNA due to homologous recombination that can take place between the
NidAT6-encoding sequences. To avoid this recombination event, those skilled in the art will

10   recognize the necessity to introduce changes in the NidAT6 DNA sequence to make a series
of modified NidAT6 DNA domains that differ in DNA sequence from each other and from
the natural NidAT DNA but which still encode a functional domain that can be used to
replace a methyl group with an hydroxyl group in erythromycin. Such derivatives of
NidAT6, when used in combination with NidAT6 or with each other to make two or more

15   AT replacements will render the hybrid PKS stable to mutation through homologous
recombination. The methods for making such modifications are well known to those of
ordinary skill in the art. Thus all derivatives of NidAT6 that encode a functional domain that
can be used to introduce hydroxyl groups into erythromycin are included in the scope of this
invention.

20   It is also understood that the NidAT6 domain can be used in combination with other
heterologous malonyl AT or ethylAT domains to introduce chemical diversity into the
erythromycin backbone. For example, the NidAT6 domain can be used to replace the
eryAT2 domain in *Sac. erythraea* strain ER720 EryAT1/LigAT1 which itself has the eryAT1
domain replaced by a malonyl AT domain from a PKS from *Streptomyces hygroscopicus* to

25   result in the production of the compound 10,12-didesmethyl-10-hydroxyerythromycin.
Similarly, the NidAT6 domain can be used to replace the eryAT2 domain in *Sac. erythraea*
strain ER720 EryAT4/NidAT5 that itself has the eryAT4 domain replaced by an ethyl AT
domain from the Nid PKS to result in the production of the compound 6,10-didesmethyl-6-
ethyl-10-hydroxyerythromycin A. Therefore, all compounds produced from the substitution

30   of two or more AT domains from the eryPKS with any combination of AT domains that
encode malonyl, ethyl or hydroxymalonyl starter domains and their corresponding strains that
produce them are included under the scope of this invention.

Furthermore, those skilled in the art will understand that the NidAT6 domain can be
used in gene replacements in *srmG*, *tylG*, the rifPKS DNA, the rapPKS DNA, or other

35   modular PKS genes, to introduce hydroxyl groups in spiramycin, tylosin, rifamycin,
rapamycin or other reduced polyketides. Therefore, the use of NidAT6, or any other AT

domain that specifies a hydroxymalonyl starter domain, such as the AT domains from the sixth modules of the carbomycin PKS, the midecamycin PKS or maridomycin PKS, as examples, to introduce hydroxyl groups at one or more positions in the polyketide backbones of erythromycin, spiramycin, rifamycin, rapamycin or any other polyketide that employs a modular PKS for its assembly is included under the scope of this invention. In addition, the use of NidAT6, or any other AT segment that specifies a hydroxymalonyl starter domain, in combination with LigAT2, or any other segment that specifies a malonyl starter domain, or in combination with NidAT5, or any other AT segment that specifies an ethylmalonyl starter domain, to make two or more replacements in the *eryA*, *srmG*, *tylG* or any other modular PKS to introduce chemical diversity into erythromycin, spiramycin, tylosin or other polyketides that employ a modular PKS for their synthesis is included under the scope of this invention.

Whereas a 3.0 kb segment of the *rapA* gene from *Streptomyces hygroscopicus* ATCC 29253 encoding the rapligase and adjacent ERS domains is exemplified herein to replace the ATs domain of the eryPKS to yield a hybrid PKS that encodes the production of the compound13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A, it is understood that several other gene replacements using longer segments of the *rapA* gene may be used in place of the 3.0 kb segment in analogous gene replacement experiments to create strains that yield the same product. Examples of longer segments include but are not limited to those that contain the rapligase - ERS segment and the adjacent ACP domain of *rapA* that can be used to replace the ATs - ACPs segment of the eryPKs and those that contain the rapligase - ERS segment and the adjacent ACP - KS1-encoding segment of *rapA* to replace the ATs - ACP - KS1 segment of the eryPKS. Thus, all segments of *rapA* that can be used in gene replacements with the *eryAI* gene to result in the synthesis of 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A, along with the strains that produce13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A are included under the scope of the present invention.

In addition, whereas the production of 13-3,4-dihdroxycyclohexylerythromycin A in *Sac. erythraea* EryATs/rapligase 3.0 was dependent upon the feeding of the compound 3,4-dihydroxycyclohexylcarboxylic acid to the culture medium, those skilled in the art understand that various salts and esters of 3,4-dihydroxycyclohexylcarboxylic acid can be used in place of 3,4-dihydroxycyclohexylcarboxylic acid to yield 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A. Furthermore, derivatives of 3,4-dihydroxycyclohexylcarboxylic acid or its corresponding salts or esters can also be fed to *Sac. erythraea* EryATs/rapligase 3.0 in place of 3,4-dihydroxycyclohexylcarboxylic acid or its salts or esters to result in the production of derivatives of 13-desethyl-13-(3',4'-

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dihydroxycyclohexyl)methylerythromycin A. Examples of derivatives of 3,4-
dihydroxycyclohexylcarboxylic acid or its salts or esters that can be fed to *Sac. erythraea*
EryATs/rapligase 3.0 include, but are not limited to, 3-hydroxycyclohexylcarboxylic acid, 4-
hydroxycyclohexylcarboxylic acid, shikimic acid, 3-methoxy-4-

5   hydroxycyclohexylcarboxylic acid, and the like to yield 13-desethyl-13-(3'-
hydroxycyclohexyl)methylerythromycin A, 13-desethyl-13-(4'-
hydroxycyclohexyl)methylcrythromycin A, 13-desethyl-13-(3',4',5'-
trihdroxycyclohexyl)methylerythromycin A, 13-desethyl-13-(3'-methoxy-4'-
hydroxycyclohexyl)methylerythromycin A, and the like, respectively. Therefore, all

10   derivatives of 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A that can be
produced by the feeding of derivatives of 3,4-dihydroxycyclohexylcarboxylic acid to *Sac.
erythraea* EryATs/rapligase 3.0 are included within the scope of the present invention.

Furthermore, those of ordinary skill understand that following the methods described
herein for replacement of resident AT-encoding DNA fragments in the eryPKS, the DNA

15   fragments encoding malonate-ATs in *S. hygroscopicus, S. venezuelae,* or *S. caelestis,* and
ethylmalonate or hydroxymalonate-ATs in *S. caelestis* may be replaced with those AT-
encoding DNA fragments from the eryPKS which utilize methylmalonyl CoA as a substrate.
As with the eryPKS, all combinations are contemplated, leading to the production of, for
example, 13-methylrapamycin, 15-methylrapamycin, 33-methylrapamycin, 13,15-

20   dimethylrapamycin, 13,15,33-trimethylrapamycin, and 10-methylpikromycin.

The methods of the present invention are widely applicable to all erythromycin-
producing microorganisms, of which a non-exhaustive list includes *Saccharopolyspora*
species, *Streptomyces griseoplanus, Nocardia* sp., *Micromonospora* sp., *Arthrobacter* sp. and
Streptomyces antibioticus. Of these, *Sac. erythraea* is the most preferred. Other hosts, which

25   normally do not produce erythromycin but into which the erythromycin biosynthesis genes
can be introduced by cloning, can also be employed. Such strains include but are not limited
to *Streptomyces coelicolor* and *Streptomyces lividans* or *Bacillus subtilis,* as examples. In
each of the other erythromycin-producing strains, replacement of the resident AT domains in
the erythromycin PKS is conducted by double homologous recombination using cloned

30   eryPKS sequences on both sides of the AT domain to be replaced to effect the switching of
the resident AT with a heterologous AT as illustrated in the Examples that follow.

Many other variations of the methods that are illustrated in the Examples that follow
will occur to those skilled in the art. For example, whereas the plasmids pUC18, pUC19,
pGEM3Zf, and pCS5 were employed in the present invention for the cloning of the LigAT2,

35   venAT, rapAT14, NidAT5, or NidAT6-encoding DNA fragments and construction of the
integration vectors, other plasmids, phage, or phagemids including but not limited to

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pBR322, pACYC184, M13mp18, M13mp19, pGEM7Zf and the like can be used in their place to allow the same constructions to be made. Furthermore, many alternative strategies can be followed for the cloning of the heterologous AT-encoding DNA fragments into integration vectors that enable homologous recombination to occur in corresponding regions of the eryPKS. Examples of alternative strategies include the use of longer or shorter fragments of DNA corresponding to either the AT domains or the flanking sequences, using different restriction sites for the cloning of the AT domains or the adjacent flanking sequences, or changing the sequence of a resident AT-encoding DNA fragment so that it expresses a domain which recognizes malonyl CoA as a substrate rather than methylmalonyl CoA. All such variations are within the scope of the present invention. Similarly, employing alternative strategies to introduce DNA into *Sac. erythraea* or other erythromycin-producing hosts for the purpose of effecting gene exchange to result in the production of novel erythromycins, such as conjugation, transduction or electroporation are also included within the scope of the present invention.

Those skilled in the art also understand that erythromycins B, C and D are naturally occurring forms of erythromycin and therefore would be produced as novel derivatives in *Sac. erythraea* by the modifications disclosed herein. Production of these forms may be further enhanced by inactivation of *eryK* (Stassi, D. *et al. J. Bacteriology* , 175:182-189, (1993)) to yield erythromycin B derivatives, *eryG* (S. F. Haydock et al. *Mol. Gen. Genet.* 230:120-128(1991)) to yield erythromycin C derivatives and *eryK* and *eryG* to yield erythromycin D derivatives. Furthermore, in *Sac. erythraea*, 6-deoxy forms of the novel erythromycins A, B, C and D can be generated by inactivation of *eryF* (J. M. Weber et al. *Science* 252:114-117(1991)) (in addition to those specified above), which encodes the hydroxylase responsible for hydroxylating the C-6 position. In addition, conversion of 6-deoxy forms of the novel erythromycins A, B, C and D to their corresponding erythromycin A, B, C, and D derivatives may be accomplished by cloning additional copies or by employing other means of overexpression of the *eryF* gene in the production host. Similarly, conversion of novel forms of erythromycins B, C and D to novel forms of erythromycin A may be achieved by expressing or overexpressing *eryK* and/or *eryG* in the production host. The methodologies for generating erythromycins B, C and D and 6-deoxyerythromycins A, B, C and D are well known to those of ordinary skill in the art.

Those skilled in the art also understand that erythronolide B and 3-α-mycarosylerythronolide B are naturally occurring intermediates in the biosynthesis of erythromycin and therefore would be produced as novel intermediates in *Sac. erythraea* by the modifications disclosed herein. Production of these forms may be further enhanced by inactivation of any of the *eryB* genes to yield erythronolide B or *eryC* genes to yield 3-α-L-

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mycarosylerythronolide B (Weber et al. *J. Bacteriol.* 172:2372-2383 (1990)) and Haydock et al. *Mol. Gen. Genet.* 230:120-128 (1991)). Furthermore, 6-deoxy forms of these novel intermediates can be generated by inactivation of *eryF* as described above. The methodologies for generating erythronolide B and 3-α-mycarosylerythronolide B, as well as

5   their 6-deoxy derivatives, are well known to those of ordinary skill in the art.

Bacterial Strains, Plasmid Vectors, and Growth Media

The erythromycin-producing microorganism used to practice the following examples of the invention was *Sac. erythraea* ER720 (J.P. DeWitt, *J. Bacteriol.* 164: 969 (1985)). The

10   host strain for the growth of *E. coli* derived plasmids was DH5α from GIBCO BRL, Gaithersburg, MD). The *S. hygroscopicus* strain that carries the Lig-PKS cluster is available from the American Type Culture Collection , Bethesda, MD under the accession number ATCC 29253. The *S. venezuelae* strain that carries the venAT domain described herein is available from the American Type Culture Collection , Bethesda, MD under the accession

15   number ATCC 15439.

E. coli bacteria carrying pUC18/venAT has been deposited at the Agricultural Research Culture Collection (NRRL), 1815 N. University Street, Peoria, Illinois 61604 U.S.A., as of December 23, 1996, under the terms of the Budapest Treaty and will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after

20   the last request for the deposit, or for the enforceable period of the U.S. patent, whichever is longer. The deposit and any other deposited material described herein are provided for convenience only, and are not required to practice the present invention in view of the teachings provided herein. The DNA sequence in all of the deposited material is incorporated herein by reference. *E. coli* bacteria carrying pUC18/venAT was accorded NRRL Deposit

25   No B-21652.

Plasmid pUC18 and pUC19 can be obtained from GIBCO BRL. Plasmid pCS5, a multifunctional vector for integrative transformation of *Sac. erythraea* is described in Vara, *et al., J. Bacteriology* , 171:5872-5881 (1989) and is referred to therein as pWHM3. Cosmid pNJ1 is described in Tuan, *et al., Gene,* 90: 21-29 (1990).

30   *Sac. erythraea* was grown for protoplast formation and routine liquid culture in 50 mL of SGGP medium (Yamamoto, *et al., J. Antibiotic.* 39:1304 (1986)), supplemented with 10 μg of thiostrepton/mL for plasmid selection where appropriate.

Reagents and General Methods

35   Commercially available reagents were used to make compounds, plasmids and genetic variants of the present invention, including butyric acid, ampicillin, thiostrepton, restriction

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endonucleases, T4-DNA ligase, and calf intestine alkaline phosphatase. The nucleotide sequence of the *eryA* genes from *Sac. erythraea* has been deposited in the GenBank database under the accession numbers M63676 and M63677 and are publicly available.

5   Standard molecular biology procedures (Maniatis *et al.*, *supra*) were used for the construction and characterization of replacement plasmids. Plasmid DNA was routinely isolated by the alkaline lysis method (H. C. Birnboim and J. Doly, 1979 Nucleic Acids Res. 7: 1513) or with QIAprep Spin Plasmid kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturers instructions. Restriction fragments were recovered from 0.8-1% agarose gels with Prep-A-Gene (BioRad). The products of ligation for each step of the plasmid

10   constructions were used to transform the intermediate host, *E. coli* DH5α (GIBCO BRL), which was cultured in the presence of ampicillin to select for host cells carrying recombinant plasmids. Selection for insert DNA with X-gal was used where appropriate. Typically, LB plates contain 30 mL of LB agar (Maniatis *et al.*, *supra*). Plasmid DNAs were isolated from individual transformants that had been grown in liquid culture and characterized with respect

15   to known restriction sites. DNA sequence determination was by cycle sequencing (*fmol* DNA Sequencing System, Promega Corp. Madison, WI) according to the manufacturer's instructions.

SCM medium consists of 20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS, 1.5 g Yeast Extract and 0.1 g $CaCl_2$ per liter of distilled H_2O. SGGP medium consists of 4 g

20   peptone, 4 g yeast extract, 4 g casamino acids, 2 g glycine, 0.5 g $MgSO_4 \cdot 7 H_2O$, 10 g glucose, 20 mL of 500 mM KH_2PO_4 per liter of aqueous solution (Yamamoto, *et al.*, 1986, J. Antibiotic. 39:1304). P_M buffer (per liter) is 200 g sucrose, 0.25 g K_2SO_4 in 890 mL H_2O, with the addition after sterilization of 100 mL 0.25 M TES, pH 7.2, 2 mL trace elements solution (Hopwood, *et al.*, 1985, Genetic Manipulation of Streptomyces A Laboratory

25   Manual, The John Innes Foundation), 0.08 mL 2.5 M $CaCl_2$, 10 mL 0.5% KH_2PO_4, 2 mL 2.5 M $MgCl_2$.

Integrative transformation of *Sac. erythraea* protoplasts, and routine growth and sporulation were carried out according to procedures described in Donadio, *et al.*, 1991, Science 115:97; Weber and Losick, 1988, Gene 68:173; and Yamamoto, *et al.*, 1986, J.

30   Antibiotic. 39:1304.

Oligo primers used in the PCR amplifications and described in the Examples below are as follows:

5'-ATCTACACSTCSGGCACSACSGGCAAGCCSAAGGG-3'	SEQ ID NO:3
5'-CTSAAGGCSGGCGGCGCSTACGTSCCSATCGACCC-3'	SEQ ID NO:4
5'-CGCGAATTCCTAGGCTGGCGGTGATGTTCA-3'	SEQ ID NO:5
5'-GCCGGATCCATGCATACGTCGGCAGGGAGGTAC-3'	SEQ ID NO:6

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5'-GCTCGAATTCGCTGGTCGCGGTGCACCT-3'	SEQ ID NO:7
5'-GACGGATCCGGCCCTAGGCTGCGCCCGGCTCG-3'	SEQ ID NO:8
5'-TTGGGATCCTATGCATTCCAGCGCGAGCGC-3'	SEQ ID NO:9
5'-GAGAAGCTTGGCGCGACTTGCCCGCT-3'	SEQ ID NO:10
5'-TTTTTTAAGCTTGGTACCTGCTCACCGGCAACACCG-3'	SEQ ID NO:11
5'-TTTTTTGGATCCCTGCAGCCTAGGGTCGGAGGCACTGCCGGT-3'	SEQ ID NO:12
5'-TTTTTTCTGCAGTATGCATTCCAGGGCAAGCGGTTCT-3'	SEQ ID NO:13
5'-TTTTTTGAATTCACGCGTTGCCCGCGGCGTAGGCGC-3'	SEQ ID NO:14
5'-GATCGAATTCCCTAGGACGGCAGTCCTGCTCACC-3'	SEQ ID NO:15
5'-GATCGGATCCATGCATACGTCGGAAGGTCGACCCG-3'	SEQ ID NO:16
5'-TTCGAAGAATTCCCTAGGGTTGCCTTCCTGTTCGAC-3'	SEQ ID NO:17
5'-TTCGAAAAGCTTATGCATAGACCGGCAGATCCACCG-3'	SEQ ID NO:18
5'-CGGTSAAGTCSAACATCGG-3'	SEQ ID NO:19
5'-GCRATCTCRCCCTGCGARTG-3'	SEQ ID NO:20
5'-GAGAGAGGAACCAACGCGCACGTGATCGTCGAAGAGGCACCAGC-3'	SEQ ID NO:21
5'-GAGAGAGGATCCGACCTAGGCGCGGAGGTCACCGGCGCGACGGCG-3'	SEQ ID NO:22
5'-GAGAGACCTAGGAAGCCGGTGTTCGTGTTCCCCGGCCAGGGCT-3'	SEQ ID NO:23
5'-GAGAGAGGATCCGAGGCCGGCCGTGCGCCCGGACCGAAGACCGCCTC-3'	SEQ ID NO:24
5'-GAGAGAATTCCCTAGGGTCGCCTTCGTCTTTCCCGGGCAGG-3'	SEQ ID NO:25
5'-TTGAGATCTTATGCATACGAGGGAAGCGGCACCCTGC-3'	SEQ ID NO:26
5'-TTTGAATTCACGTCCTCGACGTGCAGCA-3'	SEQ ID NO:35
5'-TTTGGATCCCCTAGGGGACGGCCGGGCCACGCC-3'	SEQ ID NO:36
5'-TTTGGATCCATGCATCTGCCGGAGTTCGCGCCG-3'	SEQ ID NO:37
5'-TTTAAGCTTGCGCCCGCCCGTTGGGC-3'	SEQ ID NO:38
5'-ATGGCTTCCGACAGTCCCCGCCCAAGGCCG -3	SEQ ID NO:39
5'- ACCAATTCCGTCGGCGGGCACCAGGCCACC -3'	SEQ ID NO:40
5'-TTTTGAATTCCCTAGGATGTCACGCGCGGAACTGG -3'	SEQ ID NO:41
5' -TTTTGCATGCGTCAGTGCGAGCCG -3'	SEQ ID NO:42
5'-TTTTCTCGAGGTCGGCCCGGAAGT -3'	SEQ ID NO:43
5'-TTTTAAGCTTATGCATGTCGAGTCGCCGGGGAATGG -3'	SEQ ID NO:44

Mass spectrometry was routinely performed with a Finnigan-MAT 7000 mass spectrometer equipped with an atmospheric pressure chemical ionization source (APCI). Electrospray mass spectrometry (ESI-MS) was performed with a Finnigan-MAT 752-7000 mass spectrometer equipped with a Finnigan atmospheric pressure ionization (API) source. HPLC separation was carried out on a Hewlett-Packard 1050 liquid chromatograph using a Prodigy ODS (2) column (5μm, 50x2mm) and a gradient elution of 5mM ammonium acetate and methanol. The flow rate was 0.3 mL/min.

For large scale preparation of erythromycin derivatives, fermentation beers are typically adjusted to pH 9 with NH4OH and then extracted two times with an equal volume of CH2Cl2. The pooled extract is then concentrated to a wet oil (approx. 1 g per liter of

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fermentation beer). Concentrated extracts are digested in methanol and chromatographed over a column of Sephadex® LH-20 (Pharmacia Biotech, Uppsala, Sweden) in the same solvent. Fractions are tested for bioactivity against *Staphylococcus aureus*, and active fractions are combined and concentrated. When additional column chromatography is desired to reduce sample weight, the concentrated sample is digested in a solvent system consisting of n-heptane, chloroform, ethanol (10:10:1, v/v/v) and chromatographed over a column of Sephadex® LH-20 in the same system. Fractions are then analyzed by ^{1}H NMR, focusing on the characteristic erythromycin resonances around δ = 5.0 (H-13), δ = 4.9 (H-1"), and δ = 4.4 (H-1') (Everett and Tyler, J. Chem. Soc. Perkin Trans. I, pg. 2599 (1985)) and pooled according to purity. Alternatively, column chromatography is replaced with an extraction sequence. In this case, the initial pooled CH_2Cl_2 extract is concentrated to approximately 400 mL. This is extracted twice with equal volumes of 0.05 M aqueous potassium phosphate with the pH chosen between pH 4.5-6. The aqueous phase is then pooled, adjusted to pH 8-9, and extracted twice with equal volumes of ethyl acetate. Finally, the ethyl acetate extracts are pooled and concentrated. When additional reduction in sample weight is desired, the extraction sequence is repeated on a 10-50 fold smaller scale, typically yielding about 500 mgs of partially pure material.

High resolution separation of erythromycin derivatives is obtained by one or more rounds of countercurrent chromatography (Hostettmann and Marston, *Anal. Chim. Acta*, 236:63-76 (1990)). When the weight of the partially pure sample from column chromatography or the extraction sequence is less than 5 g, but greater than 0.5 g, it is digested in 7 mL of the upper phase of a solvent system (3:7:5, v/v/v) consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, with a pH chosen between 6.5-8.0, and chromatographed on a custom droplet countercurrent chromatography (DCCC) instrument [100 vertical columns, 0.4 cm dia. x 24 cm length; Hostettmann and Marston, *Anal. Chim. Acta*, 236:63-76 (1990)] in the same system with the upper phase as the mobile phase. Flow rates of approximately 120-200 mL/hr are employed. As before, fractions are analyzed by NMR and bioactivity, and pooled according to purity. When the weight of the partially pure sample is approximately 0.5 g or less, countercurrent chromatography is carried out on an Ito multi-layered horizontal Coil Planet Centrifuge (P.C. Inc., Potomac, MD) using either the system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, with the pH chosen between 6.5-8.0, (3:7:5, v/v/v) employed above, or similar systems in which the ratio of hexane to EtOAc and/or the pH are varied. The chromatography is developed either isocratically, or with a gradient starting, for example, with the upper phase of a solvent system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, with the pH chosen between 6.5-8.0, (7:3:5, v/v/v) and finishing with the upper phase of a solvent

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system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, at the same pH, (1:1:1, v/v/v). In all cases, flow rates of approximately 120 mL/hr are employed. As before, fractions are analyzed by NMR and bioactivity, and pooled according to purity. Once sufficient purity is achieved, ^{1}H and ^{13}C NMR spectra are measured with a General

5   Electric GN500 spectrometer and structural assignments are made with the aid of with the aid of correlational spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and distortionless enhancement by polarization transfer (DEPT) experiments.

The foregoing can be better understood by reference to the following examples, which

10   are provided as non-limiting illustrations of the practice of the instant invention.

EXAMPLE 1: Cloning of the LigAT2 Domain from
Streptomyces hygroscopicus ATCC 29253

A genomic library of _Streptomyces hygroscopicus_ ATCC 29253 DNA was

15   constructed in the bifunctional cosmid pNJ1 (Tuan, _et al._, _Gene_ 90: 21-29 (1990)) using standard methods of recombinant DNA technology. Briefly, cosmid vector was prepared by digesting approximately 5 μg of pNJI with _Eco_RI, dephosphorylating with calf intestinal alkaline phosphatase (CIAP) and then digesting with _Bgl_II to generate one arm and also digesting 5 μg of pNJI with _Hind_III, dephosphorylating with CIAP and then digesting with

20   _Bgl_II to generate the other. Insert DNA was prepared by partially digesting approximately 25 μg of high molecular weight _S. hygroscopicus_ chromosomal DNA with _Sau_IIIA according to the procedure outlined in Maniatis, _et al. supra_. _Sau_IIIA fragments of approximately 35 kb were recovered from a 0.5% low melting point agarose gel by melting the appropriate gel slice to 65°C, adding 3 volumes of TE buffer, gently extracting 2X with phenol and once with

25   chloroform and ethanol precipitating the aqueous phase. For the ligation, approximately 3 μg of this chromosomal DNA was mixed with approximately 0.5 μg of each cosmid arm and EtOH precipitated. The precipitate was resuspended in 7 μL of water to which was added 2 μL of 5X ligation buffer and 1 μL of T4 DNA ligase. The mixture was incubated overnight at 16°C. GigapackII XL (Stratagene®) was used for packaging 2 μL of the ligation mix

30   according to the manufacture's instructions. The host bacterium was _E. coli_ ER1772 from New England Biolabs (Beverly, MA). Twenty-six colonies were examined by restriction analysis and all were found to contain insert DNA. Individual colonies were picked into thirty-four 96-well plates to give a 99.99% probability that the library represented all _S. hygroscopicus_ sequences. Further restriction analysis demonstrated the average insert size to

35   be about 30 kb.

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The library was screened with a 1.45 kb *Sstl-Mscl* DNA fragment encompassing the
ketosynthase (KS) domain from module 5 of the erythromycin PKS gene *eryAlll* (Donadio
and Katz, 1992, Gene, 111: 51-60). The DNA fragment was labeled with ^{32}P using the
Megaprime DNA labeling system (Amersham Life Science, Arlington Heights, IL). Colonies
5   (3600) were transferred from 96-well plates to Hybond-N nylon membranes (Amersham Life
Science, Arlington Heights, IL) and probed according to procedures outlined in Maniatis, *et
al. supra*. Hybridization was performed at 65°C and a stringency wash carried out with 0.1x
SSC at 65°C. About 60 cosmid clones were chosen which gave the strongest signals with this
PKS probe.

10   We also decided to screen Southern digests of these clones with a second probe in
order to identify potential genetically linked peptide synthetases in this strain. The probe was
designed from conserved motifs of nonribosomal peptide synthetases (Borchert *et al.*, 1992,
FEMS Microbiology Letters, 92: 175-180) and consisted of a mixture of two degenerative
35-mers, SEQ ID NO:3 and SEQ ID NO:4. The mixed probe was labeled using DNA 5' End
15   Labeling System (Promega Corp., Madison, WI). The 60 cosmid clones were digested with
*Sma*I and run on 0.9% agarose gels. Southern analysis was performed according to Maniatis,
et al. supra, except that hybridization was overnight at 55°C and the stringency wash was
with 0.5x SSC at 55°C. Two cosmids, 54 and 58, were identified using this second probe.,
Thirteen additional cosmids were subsequently isolated by re-probing the cosmid library with
20   a 1kb fragment from the left of the insert of cosmid 58. Two of these thirteen cosmids,
designated A15 and A16, were then further analyzed by restriction analysis and DNA
sequencing. Restriction and sequence analysis of a 32.8 kb continuous segment of DNA
from A16 revealed a type I PKS cluster with four PKS modules. A genetic map of the cluster
is shown in FIG. 6. Since an unusual CoA ligase-like domain was found in ORF1 (PKS1),
25   the cluster was named "Lig-PKS".

The nucleotide sequence of the LigAT2 domain from Lig-PKS (top strand) and its
corresponding amino acid sequence (bottom strand) are shown in FIG. 7 (SEQ ID NO:1 and
SEQ ID NO:31 respectively). When SEQ ID NO:31 was compared with the 14 AT domains
in the rapamycin PKS (Growtree Program, GCG, Madison WI), it was found to cluster with
30   malonate-specifying rapamycin domains (see Growtree analysis of FIG. 3). Therefore, it was
predicted that the LigAT2 specifies malonate as its cognate extender unit during synthesis of
the polyketide encoded by Lig-PKS.

EXAMPLE 2: Construction of plasmid pUC18/LigAT2

35   Two PCR oligonucleotides (SEQ ID NO:5 and SEQ ID NO:6) were designed to
subclone the 985 bp DNA segment encoding the LigAT2 domain from the Lig-PKS cluster

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and to introduce two unique restriction sites, *Avr*II and *Nsi*I, for cassette cloning. The unique restriction sites *Avr*II and *Nsi*I required for cassette cloning of the AT-encoding DNA were chosen based on multiple sequence alignment using the programs PILEUP and PRETTY (GCG, Madison WI) which compared the amino acid sequences of LigAT2, venAT, rapAT2,

5   rapAT5, rapAT8, rapAT9, rapAT11, rapAT12, rapAT14, eryAT1, eryAT2, eryAT3, eryAT4, eryAT5, eryAT6, and a monofunctional AT from *Streptomyces glaucescens* (R.G. Summers *et al., Biochemistry* 34:9389-9402 (1995)). The selection and positioning of the restriction enzyme sites were based on the following considerations: (i) extent of amino acid sequence conservation among the various ATs, with the sites being positioned outside, but near the

10   regions of greatest conservation, (ii) absence of the sites from the heterologous AT-encoding DNA and the eryAT flanking DNA and (iii) impact of the amino acid sequence changes resulting from translation of these sites on the heterologous AT amino acid sequence. This necessitated nucleotide changes, shown in bold in FIG. 8, at the beginning and near the end of the LigAT2-encoding DNA sequence. (In FIG. 8, the underlined nucleotides are the wild-type

15   sequence.) In addition, two other restriction sites, *Eco*RI and *Bam*HI, were also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb LigAT2 domain was amplified from Cosmid 58 as follows: The 100 μL PCR reaction mixture contained 10 μL of 10x PCR buffer (Bethesda Research Laboratories), 2 μL of 10 mM dNTP mixture, 2-4 μL of

20   50 mM MgCl₂, 100 pM of each oligo, 10-50 ng of template DNA and water to 100 μL. Cycling conditions were as follows: One cycle at 96°C/6 min, 80°C/1 min (add 5 U Taq DNA Polymerase during this 1 min) and 72°C/2 min; 30 cycles at 95°C/1 min, 65°C/1 min and 72°C/2 min with a 5 min extension at 72°C for the last cycle. The entire reaction was then run on a 1% agarose gel and the desired fragment was isolated with Prep-A-Gene

25   (BioRad, Hercules, CA). The PCR product was digested with *Eco*RI and *Bam*HI and subcloned into the *Eco*RI and *Bam*HI sites of pUC18. The ligation mixture was transformed into *E. coli* DH5α (GIBCO BRL) according to the manufacturer's instructions and transformants were selected on LB plates containing 150 μg/mL ampicillin and 50 μL of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis

30   and the fidelity of the insert was confirmed by DNA sequencing. The final plasmid construct was named pUC18/LigAT2.

EXAMPLE 3:   Construction of plasmid pEryAT1/LigAT2

pEryAT1/LigAT2 was constructed using standard methods of recombinant DNA

35   technology according to the schematic outlines of FIGS. 9 and 10. To construct a gene-replacement vector specific for the eryAT1 domain, the two DNA regions immediately

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adjacent to eryAT1-encoding DNA were cloned and positioned adjacent to the LigAT2-encoding DNA as described in EXAMPLE 2. The 5' and 3' boundaries of eryAT1 were designated as 3825 and 4866, and correspond to the deposited *eryAI* sequence (GenBank accession number M63676). To subclone the DNA fragment upstream of the eryAT1 domain

5 encoding region from the *Sac. erythraea* chromosome, two PCR oligonucleotides (SEQ ID NO:7 and SEQ ID NO:8) were designed so that an *EcoRI* site was added at the 5' end of the region and *AvrII-BamHI* restriction sites were introduced at the 3' end. The 5'-flanking region (about 1 kb) was PCR generated as described in EXAMPLE 2 using plasmid pAIEN22 DNA as template. (This plasmid is a pUC19 derivative containing 22 kb of *Sac. erythraea* DNA

10 from an *EcoRI* site upstream of *eryAI* to an *NheI* site in *eryAII* cloned into *EcoRI* and *XbaI* cut pUC19). The PCR product was subcloned into *EcoRI* and *BamHI* sites of pUC19 and the ligated DNA transformed into *E. coli* DH5α (GIBCO BRL) according to the manufacturer's instructions. Clones were selected on LB plates containing 150 µg/mL ampicillin and 50 µL of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction

15 analysis and the fidelity of the insert was confirmed by DNA sequencing. The resulting construct was named pUC19/AT1/5'-flank.

For subcloning the 3'-flanking region of the eryAT1 from *Sac. erythraea* chromosome, two PCR oligonucleotides (SEQ ID NO:9 and SEQ ID NO:10) were designed so that *BamHI-NsiI* restriction sites were introduced into the 5' end of the region and a

20 *HindIII* restriction site was added to the 3' end. The 3'-flanking region (about 1 kb) was also generated by PCR using pAIEN22 as template as described above. The PCR fragment was subcloned into the *BamHI* and *HindIII* sites of pUC19 and the ligated DNA transformed into *E. coli* DH5α as above. Clones were selected on LB plates containing 150 µg/mL ampicillin and 50 µL of a 2% solution of X-gal for blue/white selection. Clones were confirmed by

25 restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. This intermediate construct was named pUC19/AT1/3'-flank. The two flanking regions were joined by first isolating the 1 kb *BamHI-HindIII* fragment (3'-flank) from pUC19/AT1/3'-flank and then ligating this fragment to pUC19/AT1/5'-flank cut with *BamHI* and *HindIII*. Ligated DNA was transformed into *E. coli* DH5α and clones isolated as described. The

30 resulting plasmid was named pUC19/AT1-flank. The 2.1 kb *EcoRI* and *HindIII* fragment from pUC19/AT1-flank was then isolated and ligated to pCS5 cut with the same enzymes to generate pCS5/AT1-flank. The final step in the construction of pEryAT1/LigAT2 was to ligate the 1 kb LigAT2 fragment having *AvrII* and *NsiI* ends to pCS5/AT1-flank cut with the same enzymes to give the gene replacement/integration plasmid pEryAT1/LigAT2. All

35 ligation mixtures were transformed into the intermediate host *E. coli* DH5α and clones selected as previously described.

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EXAMPLE 4: Construction of *Sac. erythraea* ER720 EryAT1/LigAT2

        An example of a 12-desmethyl-12-deoxyerythromycin A producing microorganism
was prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase

5   domain in module 1 of the erythromycin PKS (EryAT1) of *Sac. erythraea* ER720 with a
newly discovered DNA fragment encoding a malonyl acyltransferase domain (LigAT2) from
S. hygroscopicus ATCC 29253. This was accomplished with the recombinant plasmid,
pEryAT1/LigAT2, prepared as described in EXAMPLE 3. Transformation of *Sac. erythraea*
ER720 and resolution of the integration event were carried out according to the following

10   method. *Sac. erythraea* ER720 cells were grown in 50 mL of SGGP medium for 3 days at
32°C and then washed in 10 mL of 10.3% sucrose. The cells were resuspended in 10 mL of
P_M buffer containing 1 mg/mL lysozyme and incubated at 30°C for 15-30 minutes until most
of the mycelial segments were converted into spherical protoplasts. The protoplasts were
washed once with P_M and then resuspended in 3 mL of the same buffer containing 10%

15   DMSO for storage in 200 µL aliquots at -80°C.

        Transformation was accomplished by quickly thawing an aliquot of protoplasts,
centrifuging for 15 seconds in a microfuge, decanting the supernatant, and resuspending the
protoplasts in the P_M remaining in the tube. Ten µL of DNA solution was added (3 µL of
pEryAT1/LigAT2 DNA from EXAMPLE 3 at about 1 µg/µL in 7 µL of P_M buffer) and

20   mixed with the protoplasts by gently tapping the tube. Two tenths of a mL of 25% PEG 8000
in T buffer (Hopwood, *et al.*, 1985, Genetic Manipulation of Streptomyces A Laboratory
Manual, The John Innes Institute) was then added, mixed by pipetting the solution 3 times
and the suspension immediately spread on a dried R3M plate. The plate was incubated at
30°C for 20 hours and overlaid with 2 mL of water containing 100 µg/mL thiostrepton, dried

25   briefly and incubated 4 more days at 30°C.

        To select stable transformants (integrants) colonies arising on the transformation
plates were re-streaked onto R3M plates containing thiostrepton (20 µg/mL). Two colonies
were confirmed to be thiostrepton resistant and one of these was inoculated into SGGP
containing thiostrepton (10 µg/mL) to isolate chromosomal DNA for Southern analysis.

30   Integration of the plasmid DNA into the ER720 chromosome was further confirmed by
Southern hybridization (data not shown). Hybridization was at 65°C and the stringency wash
was with 0.1x SSC at 65°C.

        The confirmed integrant was grown in SGGP without antibiotic for four days and then
plated onto non-selective R3M plates for sporulation. Spores were plated on R3M plates to

35   obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating
loss of the plasmid sequence from the chromosome. Five thiostrepton sensitive colonies were

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selected and their chromosomal DNA digested with *Sph*I and analyzed by Southern
hybridization. Hybridization was at 65°C and the stringency wash was with 0.1x SSC at
65°C. In three of the five thiostrepton sensitive colonies, a probe consisting of an
approximately 3 kb *Eco*RI/*Hind*III fragment from pEryAT1/LigAT2 hybridized with

5   fragments of approximately 3.5 and 1.6 kb, indicating that LigAT2 had replaced EryAT1 in
the chromosomes of these resolvants. The strain was named *Sac. erythraea* ER720
EryAT1/LigAT2.

       EXAMPLE 5: Analysis of compounds produced by *Sac. erythraea* ER720 EryAT1/LigAT2
10   ·   Compounds produced by the recombinant *Sac. erythraea* strain, ER720
EryAT1/LigAT2, whose construction is described in EXAMPLE 4, were characterized by
TLC, bioautography, mass spectrometry and NMR analysis.
            For TLC analysis cells were grown in either SGGP or SCM medium for 4-5 days at
30°C. An aliquot of culture (1.5 mL) was centrifuged for 1 minute in a microfuge to remove

15   cells. One mL of the resulting supernatant was removed to another microfuge tube and the
pH adjusted to 9.0 by the addition of 6 µL of NH4OH. Then 0.5 mL of ethyl acetate was
added, the tube was vortexed for 10 sec and then centrifuged for approximately 5 min to
achieve phase separation. The organic phase was removed to another tube, and the aqueous
phase was re-extracted with 0.5 mL of ethyl acetate. The second organic phase was

20   combined with the first and dried in a Speed Vac. The residue was taken up in 10 µL of ethyl
acetate and 5 µL was spotted onto a Merck 60F-254 silica gel TLC plate. The plate was run
in isopropyl ether:methanol:NH4OH (75:35:2). Erythromycin derivatives were visualized by
spraying the plates with anisaldehyde:sulfuric acid:ethanol (1:1:9). Using this reagent, a
novel compound predicted to be 12-desmethyl-12-deoxyerythromycin A, appeared as a blue

25   spot running slightly faster than erythromycin A.
            To detect biological activity, a TLC-bioautography assay was performed. In this
assay, one microliter of the extracted sample from above was spotted onto a TLC plate which
was run as described above. The plate was then air-dried and placed in a sterile bio-assay
dish (245x245x25 mm). The plate was then covered with 100 mL of antibiotic medium 11

30   (DIFCO-BACTO) containing *Staphylococcus aureus* as an indicator strain and incubated
overnight at 37°C. As with the positive controls, a clear zone of inhibition developed around
the sample spot indicating that the novel compound had bioactivity.
            To determine whether the novel spot seen on TLC had the molecular mass
corresponding to the predicted 12-desmethyl-12-deoxyerythromycin A, an ethyl acetate

35   extract was further analyzed by mass spectrometry. The mass spectrometry samples were
isolated by TLC basically as described above except that plates were not sprayed with the

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anisaldehyde reagent. The region of the novel spot was instead scraped from the TLC plate
and the silica resin re-extracted with ethyl acetate-methanol (1:1) and then twice with ethyl
acetate. The combined solvent phases were then dried in a Speed Vac. Mass spectrometric
analysis revealed the novel compound to have a mass of 704, which corresponds to the
5   molecular ion plus a proton $(M+H^+)$ of 12-desmethyl-12-deoxyerythromycin A.

To acquire milligram quantities of highly purified material for performance of NMR
analysis, the culture was grown in a 42-liter LH Fermentation Series 2000 fermentor. SCM
medium was used for growth of inoculum and for the fermentation. Seed for the
fermentation was grown in two steps. In the first step, frozen vegetative inoculum was used
10   to seed 100 mL of SCM medium in a 500 mL Erlenmeyer flask. For the second step, 2-liter
Erlenmeyer flasks containing 600 mL of SCM medium were seeded at 5% from the first
passage growth. Each step was incubated for 3 days at 32 °C on a rotary shaker operated at
225 rpm.

Thirty liters of SCM medium were prepared in the 42-liter fermentor and sterilized at
15   121°C and 15 psi for 1 hour. Antifoam (XFO-371, Ivanhoe Chemical Co., Mundelein, IL)
was added initially at 0.01% and then was available on demand. The fermentor was
inoculated with 1.5 liters of the second passage seed growth. The temperature was controlled
at 32°C. The agitation rate was 260 rpm and the air flow was 1.3 vol/vol/min. The head
pressure was maintained at 6 psi. During fermentation pH was controlled at 7.3 with 5 M
20   propionic acid. The fermentation was terminated at 111 hours, and the fermentation beer was
adjusted to pH 8. This was followed by two extractions with equal volumes of CH_2Cl_2. The
pooled CH_2Cl_2 extract was then concentrated to approximately 400 mL and extracted twice
with equal volumes of 0.05 M aqueous potassium phosphate pH 5.5. The aqueous phase was
pooled and adjusted to pH 8, and then extracted twice with equal volumes of ethyl acetate.
25   The ethyl acetate extracts were pooled and concentrated to yield 5 ml oil. The extraction
sequence described above was then repeated to yield 600 mg of oil after concentration. Next,
the sample was split and each half was digested in 2.5 ml each of the upper and lower phases
of a solvent system consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium
phosphate, pH 8, (1:1:1, v/v/v). These were then chromatographed on the Coil Planet
30   Centrifuge using the upper phase as the mobile phase. Fractions were analyzed by bioassay
against *Staphylococcus aureus* and ^{1}H NMR. Two macrolide containing peaks of bioactivity
were observed in both samples, and the later eluting peaks from each sample, which
contained most of the bioactivity, were pooled and concentrated. The concentrated material
was then digested in 2.5 mL each of the upper and lower phases of a solvent system
35   consisting of n-hexane, ethyl acetate, 0.02 M aqueous potassium phosphate, pH 6.5, (6:4:5,
v/v/v), and was chromatographed on the Coil Planet Centrifuge using the upper phase as the

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mobile phase. Fractions were analyzed by bioassay and ^{1}H NMR. Two macrolide containing
peaks of bioactivity were observed and the later eluting species was readily characterized by
its ^{1}H and ^{13}C NMR spectra as 12-desmethyl-12-deoxyerythromycin A. Parameters from
the ^{1}H NMR spectra are listed in Table 2. The assignments were made with the aid of
5   correlational spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC),
heteronuclear multiple bond correlation (HMBC), and distortionless enhancement by
polarization transfer (DEPT) experiments. Mass spectral data of this sample was also
consistent with the structural assignment. Electrospray ionization (ESI) of this sample
revealed an M+H$^+$ ion at *M/Z* 704, which is in full accord with erythromycin A lacking both
10   a methyl group and a hydroxyl group.

Table 2

^{1}H NMR chemical shift ( ) assignments for 12-desmethyl-12-deoxyerythromycin A
in CDCl$_3$

15

2-H	2.74	1'-H	4.47
3-H	4.15	2'-H	3.25
4-H	2.01	3'-H	2.49
5-H	3.58	4'-H$_a$	1.67
7-H$_a$	1.91	4'-H$_b$	1.23
7-H$_b$	1.66	5'-H	3.54
8-H	2.86	6'-H$_3$	1.23
10-H	2.70	N(CH$_3$)$_2$	2.30
11-H	4.05	1"-H	4.85
12-H$_a$	1.71	2"-H$_a$	2.40
12-H$_b$	1.46	2"-H$_b$	1.59
13-H	5.06	4"-H	3.03
14-H$_2$	1.59	5"-H	4.04
15-H$_3$	0.89	6"-H$_3$	1.30
2-CH$_3$ 1.19		3"-CH$_3$	1.25
4-CH$_3$ 1.13		OCH$_3$	3.33
6-CH$_3$ 1.38			
8-CH$_3$ 1.19			
10-CH$_3$	1.11		

35

EXAMPLE 6: Construction of plasmid pEryAT2/LigAT2

pEryAT2/LigAT2 was constructed using standard methods of recombinant DNA
technology. To make a gene-replacement vector specific for the eryAT2 domain, two DNA
regions flanking eryAT2 were cloned and positioned adjacent to the DNA encoding the
40   domain to be inserted in order to effect homologous recombination. Boundaries of the AT2
domain were chosen as described in EXAMPLE 2. The 5' and 3' boundaries of eryAT2 are

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designated as 8255 and 9282, respectively, and correspond to deposited *eryAI* sequence
(GenBank accession number M63676). To subclone the DNA fragment upstream of the
eryAT2 DNA, two PCR oligonucleotides (SEQ ID NO:11 and SEQ ID NO:12) were
designed so that a *Hind*III site was added at the 5' end of the region and *Avr*II-*Pst*I restriction

5   sites were introduced at the 3' end. For subcloning the 3'-flanking region of eryAT2, two
PCR oligonucleotides (SEQ ID NO: 13 and SEQ ID NO:14) were designed so that *Pst*I-*Nsi*I
restriction sites were introduced at the 5' end of the region and an *Eco*RI site at the 3' end.
Both the 5'-flanking and 3'-flanking regions (about 1 kb each) were PCR generated as
described in EXAMPLE 3. In the case of the 5'-flanking region, the PCR product was

10 subsequently subcloned into *Hind*III and *Pst*I sites of pUC18 whereas the PCR product of the
3'-flanking region was subcloned into the *Pst*I and *Eco*RI sites of pUC18. Ligations,
transformations and confirmations of selected clones were performed as in EXAMPLE 3.
The resulting construct containing the AT2 5'-flanking region was designated pUC18/AT2/5'-
flank and the construction containing the AT2 3'-flanking region was designated

15 pUC18/AT2/3'-flank. The two flanking regions were then joined by first isolating the 1 kb
*Pst*I and *Eco*RI fragment (3'-flank) from pUC18/AT2/3'-flank, and ligating this fragment to
pUC18/AT2/5'-flank cut with *Pst*I and *Eco*RI. The ligation was transformed into *E. coli*
DH5α and clones isolated as described. The resulting plasmid was named pUC18/AT2-
flank (FIG. 11). The 2.2 kb *Eco*RI and *Hind*III fragment from pUC18/AT2-flank was then

20 isolated and ligated to pCS5 cut with the same enzymes to generate pCS5/AT2-flank. The
final step in the construction of pEryAT2/LigAT2 was to ligate the LigAT2 encoding DNA
fragment from pUC18/LigAT2 having *Avr*II and *Nsi*I ends (described in EXAMPLE 2) to
pCS5/AT2-flank cut with the same enzymes to give the gene replacement, integration
plasmid pEryAT2/LigAT2 (FIG. 12). All ligations were transformed into the intermediate

25 host *E. coli* DH5α and clones selected as previously described.

EXAMPLE 7: Construction of *Sac. erythraea* ER720 EryAT2/LigAT2

An example of a 10-desmethylerythromycin A and 10-desmethyl-12-
deoxyerythromycin A producing microorganism was prepared by replacing the

30 methylmalonyl acyltransferase domain of module 2 of the erythromycin PKS (EryAT2) of
Sac. erythraea ER720 with a newly discovered malonyl acyltransferase domain (LigAT2)
from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant plasmid,
pEryAT2/LigAT2, prepared as described in EXAMPLE 6. Transformation of ER720 and
selection and confirmation of stable resolvants were carried out essentially as described in

35 EXAMPLE 4. Two thiostrepton sensitive colonies were selected and their chromosomal
DNA cut with *Sph*I and analyzed by Southern hybridization. In one of the two thiostrepton

sensitive colonies, a probe consisting of an approximately 1 kb LigAT2 sequence hybridized with a chromosomal DNA fragment of approximately 900 bp, indicating that LigAT2 had replaced EryAT2 in the chromosome of this resolvant. The strain was named *Sac. erythraea* ER720 EryAT2/LigAT2.

EXAMPLE 8: Analysis of compounds produced by *Sac. erythraea* ER720 EryAT2/LigAT2

Compounds produced by the recombinant *Sac. erythraea* strain, ER720 EryAT2/LigAT2, whose construction is described in EXAMPLE 7, were characterized by TLC, bioautography and mass spectrometry.

For small scale analysis, the cells were grown in either SGGP or SCM medium for 4-5 days at 30°C. The culture was processed for TLC analysis essentially as described in EXAMPLE 5. Two novel compounds predicted to be 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A, appeared as blue spots with the lower spot running slightly slower than erythromycin A and upper spot running slightly faster than erythromycin A.

To detect biological activity, a TLC-bioautography assay was performed essentially as described in EXAMPLE 5. In this assay, 0.2 to 1 microliter of the extracted sample from above was spotted onto a TLC plate which was run as described. The plate was then air-dried and placed in a sterile bio-assay dish (245x245x25 mm). The plate was then covered with 100 mL of antibiotic medium 11 (DIFCO-BACTO) containing *Staphylococcus aureus* as an indicator strain. The inhibition zones were developed by overnight incubation of the plate at 37 °C. As with the positive controls, a zone of inhibition developed around the two novel spots (compounds) indicating that each have bioactivity against *Staphylococcus aureus*.

To determine whether the novel spots seen on TLC had the molecular masses corresponding to the predicted 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract was further analyzed by mass spectrometry. The mass spectrometry samples were isolated by TLC similarly to the method described above except that plates were not sprayed with the anisaldehyde reagent. Instead, two regions which contain the novel spots were scraped from the TLC plate and the silica resin re-extracted with ethyl acetate-methanol (1:1) and then twice with ethyl acetate. The combined solvent phases were then dried in a Speed Vac. In addition to the samples described above, a crude ethyl acetate extract was also analyzed by LC-MS, in which the sample components were first separated by liquid chromatography and then analyzed by mass spectrometry. Mass spectrometric analysis revealed the two novel compounds to have masses of 720 and

704, which correspond to the molecular ion plus a proton (M+H⁺) of 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A, respectively.

EXAMPLE 9: Cloning of the venAT Domain from *Streptomyces venezuelae*

A genomic library of *Streptomyces venezuelae* ATCC 15439 DNA was constructed in the bifunctional cosmid pNJ1 (Tuan, *et al.*, *Gene* 90: 21-29 (1990)) using standard methods of recombinant DNA technology. A cosmid from this library, pVen17, was characterized by Southern analysis and *Sst*I fragments of approximately 3.5, 3.8, and 4.0 kb were found to hybridize to a 1.37 kb *Sma*I fragment that encompasses the ketosynthase (KS) domain from module 2 of the erythromycin PKS gene *eryAI* (Donadio *et al.*, *Science* 252: 675-679 (1991)). The 4.0 kb *Sst*I fragment was then subcloned into pUC19 to give pVen4.0. The nucleotide sequence of pVen4.0 insert DNA was determined from single strand DNA templates prepared from M13mp18 and M13mp19 (Yanisch-Perron, *et al.*, *Gene* , 33:103 (1985)) subclones using Sequenase version 2.0 with 7-deaza-dGTP (United States Biochemical, Cleveland, OH) and 5'-[α-³²P] or 5'-[α-³³P]-dCTP (NEN Research Products, Boston, MA). Because pVen4.0 did not contain the entire AT domain, the nucleotide sequence was extended using pVen17 DNA as the template. The nucleotide sequence of the venAT domain (SEQ ID NO:2) and its corresponding amino acid sequence (SEQ ID NO:32) is shown in FIG. 13 (top and bottom strands respectively).

EXAMPLE 10: Construction of plasmid pEryAT1/venAT

pEryAT1/venAT was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 14 and 15. Two PCR oligonucleotides (SEQ ID NO:15 and SEQ ID NO:16) were designed to subclone the 1.03 kb DNA fragment that encodes the venAT domain (FIG. 14) from the *S. venezuelae* PKS cluster and to introduce two unique restriction sites, *Avr*II and *Nsi*I, for cassette cloning (described in EXAMPLE 2). This necessitated nucleotide changes (shown in bold in FIG. 14) at the beginning and near the end of the venAT sequence (underlined nucleotides are the wild-type sequence). In addition, two other restriction sites, *Eco*RI and *Bam*HI, were also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb venAT-encoding DNA was PCR amplified from cosmid pVen17 template DNA (EXAMPLE 2) using Vent_R® DNA Polymerase (New England Biolabs). A typical PCR reaction contained 10 μL ThermoPol Buffer, 10 μL formamide, 10 μL of 20% glycerol, 55 μL water, 100 pmole of each primer, and approximately 0.2 μg DNA. The sample was heated to 99°C for 2 minutes, and then allowed to cool to 80°C for 2 minutes, at which time 16 μL of a 1.25 mM mixture of dATP,

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dCTP, dGTP, and dTTP and 2 units of Vent DNA polymerase were added. A temperature cycle of 35 seconds at 96.5°C and 2 minutes 15 seconds at 72°C was then repeated 30 times, followed by a 3 minute incubation at 72°C. The desired PCR fragment was then isolated from low melting agarose by standard procedures. The PCR product was ligated to *Hinc*II

5   digested pUC18 and transformed into *E. coli* DH5α (GIBCO BRL) according to the manufacturer's instructions. Clones were selected on LB plates containing 150 µg/mL ampicillin and 50 µL of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. The final construct was named pUC18/venAT.

10   The final step in the construction of pEryAT1/venAT was to ligate the 1 kb venAT fragment having *Avr*II and *Nsi*I ends to pCS5/AT1-flank (EXAMPLE 3) cut with the same enzymes to give the gene replacement/integration plasmid pEryAT1/venAT (FIG. 15). All ligations were transformed into the intermediate host *E. coli* DH5α and clones selected as previously described.

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EXAMPLE 11: Construction of *Sac. erythraea* ER720 EryAT1/venAT

A 12-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the methylmalonyl acyltransferase domain of module 1 of the erythromycin PKS (EryAT1) of *Sac. erythraea* ER720 with a newly discovered malonyl acyltransferase domain

20   (venAT) from *S. venezuelae* ATCC 15439 This was accomplished with the recombinant plasmid, pEryAT1/venAT, prepared as in EXAMPLE 10. Transformation of ER720 and selection and confirmation of stable resolvants were carried out essentially as described in EXAMPLE 4. Four thiostrepton sensitive colonies were selected and their chromosomal DNA cut with *Pvu*II and analyzed by Southern hybridization. In two of the four thiostrepton

25   sensitive colonies, a probe of venAT sequence hybridized with chromosomal DNA fragments of approximately 4.2 and 2.4 kb, indicating that venAT had replaced EryAT1 in the chromosomes of these resolvants. The strain was named *Sac. erythraea* ER720 EryAT1/venAT.

30   EXAMPLE 12: Analysis of compounds produced by

Sac. erythraea ER720 EryAT1/venAT

Compounds produced by the recombinant *Sac. erythraea* strain, ER720 EryAT1/venAT, whose construction is described in EXAMPLE 11, were characterized by TLC, bioautography, and mass spectrometry.

35   For TLC analysis cells were grown in either SGGP or SCM medium for 4-5 days at 30°C. The culture was processed for TLC essentially as described in EXAMPLE 5. A novel

compound predicted to be 12-desmethyl-12-deoxyerythromycin A, appeared as a blue spot running slightly faster than erythromycin A.

To detect biological activity, a TLC-bioautography assay was performed essentially as described in EXAMPLE 5. As with the positive controls, a clear zone of inhibition developed around the sample spot indicating that the novel compound was bioactive.

To determine whether the novel spot seen on TLC had the molecular mass corresponding to the predicted 12-desmethyl-12-deoxyerythromycin A, an ethyl acetate extract was further analyzed by mass spectrometry. The mass spec samples were isolated by TLC basically as described above except that plates were not sprayed with anisaldehyde. The region of the novel spot was instead scraped from the TLC plate and the silica resin re-extracted with ethyl acetate-methanol (2:1) and then twice with ethyl acetate. The combined solvent phases were then dried in a Speed Vac. Mass spectrometric analysis revealed the novel compound to have a mass of 704, which corresponds to the molecular ion plus a proton $(M+H^+)$ of 12-desmethyl-12-deoxyerythromycin A.

EXAMPLE 13: Construction of plasmid pUC19/rapAT14

Two PCR oligonucleotides (SEQ ID NO:17 and SEQ ID NO:18) were designed to subclone the 1023 bp rapAT14-encoding DNA fragment from the rapamycin biosynthetic gene cluster (GenBank Accession #: X86780) and to introduce two unique restriction sites, *Avr*II and *Nsi*I, for cassette cloning (described in EXAMPLE 2). This necessitated nucleotide changes (shown in bold in FIG. 16) at the beginning and near the end of the rapAT14 sequence. (In FIG. 16, the underlined nucleotides are the wild-type sequence.) In addition, two other restriction sites, *Eco*RI and *Hin*dIII, were also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb rapAT14-encoding DNA was amplified by PCR using chromosomal DNA from *Streptomyces hygroscopicus* ATCC 29253 as template. The PCR conditions were as follows: The 100 µL reaction mixture contains 10 µL of 10x Thermopol Buffer (New England Biolabs), 2% glycerol, 10% formamide, 100 pmoles of each oligo, 100-200 ng of template DNA and water to 84 µL. The sample was then heated to 99°C for two minutes followed by cooling to 80°C for two minutes at which time 16 µL of a dNTP solution (1.25 mM dATP and dTTP, 1.5 mM dCTP and dGTP) and 1 µL of VentR® DNA Polymerase (New England Biolabs) was added. Cycling was as follows: 30 cycles at 96.5°C/35 sec, 65°C/1 min and 72°C/1.5 min followed by one cycle at 72°C for 3 min. The entire reaction was then run on a 1.2% low-melting agarose gel and the desired fragment was isolated by melting the appropriate gel slice at 65°C, adding 3 volumes of TE buffer, extracting 2X with phenol and once with chloroform, and ethanol precipitating the aqueous

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phase. The isolated DNA was ligated directly into *Hinc*II digested pUC19. The ligation mixture was transformed into *E. coli* DH5α (GIBCO BRL) according to the manufacturer's instructions and transformants were selected on LB plates containing 150 µg/mL ampicillin and 50 µL of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. The final plasmid construct was named pUC19/rapAT14.

EXAMPLE 14: Construction of plasmid pEryAT1/rapAT14

pEryAT1/rapAT14 was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 16 and 17. To make a gene-replacement-vector specific for the eryAT1 domain, the two DNA regions immediately adjacent to eryAT1 were cloned and positioned adjacent to the DNA encoding the rapAT14 domain in order to allow homologous recombination to occur. The strategy and protocol for constructing the intermediate plasmid containing the flanking regions, pCS5/AT1-flank, are described in EXAMPLE 3 and FIG. 9. To insert the rapAT14 fragment between the flanking regions, pUC19/rapAT14 (from EXAMPLE 13) was digested with *Nsi*I and *Avr*II and the resulting 1 kb fragment was isolated from a 0.8% agarose gel with Prep-A-Gene. pCS5/AT1-flank was also digested with these enzymes and the linearized plasmid was isolated from 0.8% agarose gel. The two fragments were ligated, transformed into the intermediate host *E. coli* DH5α and ampicillin resistant clones were selected as previously described. Insertion of the rapAT14 fragment between the ery flanking regions was confirmed by restriction analysis and the resulting plasmid was called pEryAT1/rapAT14.

EXAMPLE 15: Construction of *Sac. erythraea* ER720 EryAT1/rapAT14

An example of a 12-desmethyl-12-deoxyerythromycin A producing microorganism was prepared by replacing the methylmalonyl acyltransferase domain of module 1 of the erythromycin PKS (EryAT1) of *Sac. erythraea* ER720 with the acyltransferase domain from module 14 of the rapamycin PKS from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant plasmid, pEryAT1/rapAT14, prepared as described in EXAMPLE 14. Transformation of *Sac. erythraea* ER720 and selection and confirmation of stable resolvants were carried out essentially as described in EXAMPLE 4. Six thiostrepton sensitive colonies were selected and their chromosomal DNA cut with *Sty*I and analyzed by Southern hybridization. In one of the six thiostrepton sensitive colonies, a probe consisting of an *EcoR-Hind*III fragment from pCS5AT1-flank hybridized with a chromosomal DNA fragment of approximately 1.6 kb, indicating that rapAT14 had replaced EryAT1 in the

chromosome of this resolvant. The strain was named *Sac. erythraea* ER720
EryAT1/rapAT14.

EXAMPLE 16: Analysis of compounds produced by *Sac. erythraea* ER720 EryAT1/rapAT14

Compounds produced by the recombinant Sac. erythraea strain, ER720
EryAT1/rapAT14, whose construction is described in EXAMPLE 15, were characterized by
TLC and mass spectrometry. For TLC analysis strain 4-A-1 was grown in SCM medium for
4 days at 30°C. The culture was processed for TLC essentially as described in EXAMPLE 5.
A novel compound predicted to be 12-desmethyl-12-deoxyerythromycin A, appeared as a
blue spot running slightly faster than erythromycin A.

To determine whether the novel spot seen on TLC has the molecular mass
corresponding to the predicted 12-desmethyl-12-deoxyerythromycin A, an ethyl acetate
extract was further analyzed by Mass Spectrometry. *Sac. erythraea* ER720 EryAT1/rapAT14
was grown in SCM medium for 4 days. Ten mL of culture was centrifuged to remove
mycelia and pH of the supernatant was adjusted to 9 with NH4OH. The supernatant was then
extracted twice with ethyl acetate and the organic phases pooled and dried. Mass
spectrometric analysis of this crude ethyl acetate extract shows the mass of the novel spot to
be 704, which corresponds to the molecular ion plus a proton $(M+H^+)$ of 12-desmethyl-12-
deoxyerythromycin A.

EXAMPLE 17: Construction of plasmid pEryAT2/rapAT14

pEryAT2/rapAT14 was constructed using standard methods of recombinant DNA
technology according to the schematic outlines of FIGS. 16 and 18. To make a gene-
replacement-vector specific for the *ery* AT2 domain, the two DNA regions immediately
adjacent to *ery* AT2 were cloned and positioned adjacent to the DNA encoding the rapAT14
domain in order to allow homologous recombination to occur. The strategy and protocol for
constructing the intermediate plasmid containing the flanking regions, pCS5/AT2-flank, are
described in EXAMPLE 6 and FIG. 12. The final step in the construction of
pEryAT2/rapAT14 was to ligate the 1 kb rapAT14-encoding DNA fragment having *Avr*II and
*Nsi*I ends to pCS5/AT2-flank (EXAMPLE 6) cut with the same enzymes to give the gene
replacement/integration plasmid pEryAT2/rapAT14 (FIG. 18). All ligations were
transformed into the intermediate host *E. coli* DH5α and clones selected as previously
described.

EXAMPLE 18: Construction of *Sac. erythraea* ER720 EryAT2/rapAT14

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A 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A
producing microorganism was prepared by replacing the DNA fragment encoding the
methylmalonyl acyltransferase domain of module 2 of the erythromycin PKS (EryAT2) of
Sac. erythraea ER720 with a DNA fragment encoding a malonyl acyltransferase domain

5   (rapAT14) from *S. hygroscopicus* ATCC 29253 This was accomplished with the
recombinant plasmid, pEryAT2/rapAT14, prepared as described in EXAMPLE 17.
Transformation of ER720 and selection and confirmation of stable resolvants were carried
out essentially as described in EXAMPLE 4. Four thiostrepton sensitive colonies were
selected and their chromosomal DNA cut with *Bsp*El and analyzed by Southern

10   hybridization. In three of the four thiostrepton sensitive colonies, a probe consisting of a
fragment of 5'-flanking region of eryAT2 hybridized with a chromosomal DNA fragment of
approximately 4.3 kb, indicating that rapAT14 had replaced EryAT2 in the chromosomes of
these resolvants. The strain was named *Sac. erythraea* ER720 EryAT2/rapAT14.

15   EXAMPLE 19: Analysis of compounds produced by
                                 Sac. erythraea ER720 EryAT2/rapAT14
Compounds produced by the recombinant *Sac. erythraea* strain, ER720
EryAT2/rapAT14, whose construction is described in EXAMPLE 18, were characterized by
TLC, bioassay, and mass spectrometry.

20   For TLC analysis cells were grown in either SGGP or SCM medium for 4-5 days at
30°C. The culture was processed for TLC essentially as described in EXAMPLE 5. Two
novel compounds predicted to be 10-desmethylerythromycin A and 10-desmethyl-12-
deoxyerythromycin A, appeared as blue spots with the lower spot running slightly slower
than erythromycin A and upper spot running slightly faster than erythromycin A.

25   To detect biological activity, a bioassay was performed essentially as described in
EXAMPLE 5. As with the positive controls, inhibition zones developed around the novel
compounds indicating that they have bioactivity.

            To determine whether the novel spots seen on TLC have the molecular mass
corresponding to the predicted 10-desmethylerythromycin A and 10-desmethyl-12-

30   deoxyerythromycin A, an ethyl acetate extract from another culture was further analyzed by
mass spectrometry. The sample was a crude extract of a 20 mL culture grown for 4 days.
Mass spectrometric analysis revealed the two novel compounds to have masses of 720 and
704, which correspond to the molecular ion plus a proton $(M+H^+)$ of 10-
desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A, respectively.

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                     EXAMPLE 20: Cloning of the ethylAT Domain from *Streptomyces caelestis*

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A genomic library of *Streptomyces caelestis* NRRL-2821 (U.S. Patent 3,218,239 issued November 16, 1965) DNA was constructed in the bifunctional cosmid pNJ1 (Tuan, *et al.*, *Gene*, 90: 21-29 (1990)). Cosmid vector was prepared by digesting 5 µg of pNJ1 with *Eco*RI, dephosphorylating with CIAP and then digesting with *Bgl*II to generate one arm and

5   also digesting 5 µg of pNJ1 with *Hind*III, dephosphorylating with CIAP and then digesting with *Bgl*II to generate the other. Insert DNA was prepared by partially digesting approximately 5 µg of chromosomal *S. caelestis* NRRL-2821 DNA with *Sau*IIIA according to the procedure outlined in Maniatis *et al.*, *supra*. Digestion conditions were chosen which produced fragment sizes of approximately 40 kb. The ligation was performed by mixing

10   approximately 1 µg of the digested chromosomal DNA with 0.5 µg of each cosmid arm. The ligation was incubated at 16°C overnight. GigapackII XL (Stratagene®) was used for packaging 2 µL of the ligation mix according the manufacturer's instructions. Transformation was done in *E. coli* XL1-Blue MR cells (Stratagene®). Individual colonies were picked into thirty 96-well plates to give a 99.99% probability that the library represented

15   all *S. caelestis* NRRL-2821 genomic sequences.

The library was screened using a probe specific for the *S. caelestis* NRRL-2821 PKS region. The probe was generated by PCR amplification of *S. caelestis* NRRL-2821 genomic DNA using degenerate primers designed from consensus ketosynthase (KS) and acyltransferase (AT) sequences in the GenBank database. The KS specific oligo (SEQ ID

20   NO:19) and the AT specific oligo (SEQ ID NO:20) generated a 900 bp PCR fragment. The PCR reaction contained 10 µL ThermoPol Buffer, 2 µL formamide, 25 µL of 20% glycerol, 3 µL 50 mM $MgCl_2$, 45 µL water, 50 pmole of each primer, and approximately 0.2 µg DNA. The sample was heated to 99°C for 5 minutes, and then placed on ice, at which time a 10 µL cocktail consisting of 2 µL of a 10 mM mixture of dATP, dCTP, dGTP, and dTTP, 2 units of

25   Vent DNA polymerase, and 7 µL of water was added. The sample was then transferred to a GeneAmp 9600 thermocycler (Perkin Elmer, Foster City, CA) and a temperature cycle of 1 minute at 95°C, 4 minutes at 50°C, and 4 minutes at 72°C was repeated 30 times, followed by a 15 minute incubation at 72°C. The desired PCR fragment was then isolated from 1.0% low melting agarose by standard procedures. The KS/AT probe was made by labeling

30   approximately 50 ng of the PCR fragment with ^{32}P using the Megaprime DNA Labeling System (Amersham Life Science, Arlington Heights, IL). Library clones (2,880) were transferred from the 96-well plates to Hybond-N nylon filters (Amersham) and screened with the KS/AT probe according to procedures in Maniatis, *et al.*, *supra*. Hybridization was performed at 65°C and the final wash was in 0.1x SSC at 65°C. Nineteen of the clones

35   hybridized strongly with the probe. These clones were then digested with *Sst*I, run on a 1.0% agarose gel and transferred to Hybond-N nylon filters for Southern analysis using the KS/AT

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probe. A cosmid named as pCEL18h5 was chosen for further analysis since it contained the largest number of hybridizing restriction fragments.

The *Sst*I fragments from cosmid pCEL18h5 were cloned into pGEM-3Zf (Promega, Madison, WI) and sequenced using the fmole DNA Cycle Sequencing System (Promega).
5   The reactions were run on a Sequi-Gen II Sequencing Apparatus (Bio-Rad, Hercules, CA). Individual fragments were oriented relative to one another by sequencing off of cosmid pCEL18h5 using primers that hybridized to the 5' and 3' ends of the fragments to generate upstream and downstream sequence. These sequences were then matched with sequences from the individual fragments to place them in the proper order. A very large *Sst*I fragment
10   (>10 kb) was further digested with *Sma*I to generate smaller fragments for cloning and sequencing.

By searching the GenBank database with the sequences obtained it was possible to identify the various enzymatic motifs associated with the niddamycin PKS cluster and to group these motifs into modules (see FIG. 19) based on previous knowledge of Type I PKS
15   organization. The C-6 position of the niddamycin macrolactone ring has an aldehyde derived from an ethyl side chain (FIG. 20). It was thus predicted that the AT of module 5 of the niddamycin cluster is responsible for incorporating this ethyl group into the growing chain. In addition, the carbon at C-7 of the molecule is completely saturated leading to the prediction that ER and DH motifs would also be present in module 5. These motifs were, in
20   fact, found at the predicted region of the sequence. Furthermore, motifs for the preceding 4 modules were as predicted, with an inactive ketoreductase motif in module 4 which leaves a keto group at C-9 of the ring. Sequencing of that KR showed that the nucleotide binding site GXGXXG (SEQ ID NO:27) was mutated to DXTXXP (SEQ ID NO:28). The nucleotide sequence (SEQ ID NO:29) and corresponding amino acid sequence (SEQ ID NO:33) of the
25   ethyl AT of module 5 are shown in FIG. 21 (top and bottom strands respectively).

A knockout experiment was also performed on this cluster, demonstrating that this sequence of DNA encodes the pathway for niddamycin biosynthesis.

EXAMPLE 21: Construction of plasmid pEAT4
30   A multistep strategy was used to construct the plasmid pUC/ethAT/C6 (FIG. 22), which consists of the DNA encoding the NidAT5 domain flanked by approximately 2.0 kb of sequence upstream and downstream from the eryAT4 encoding sequences, all contained in pUC19. EryAT4 flanking DNA was subcloned from pAIBX85. This plasmid is a pCS5 derivative containing 8.4 kb of *Sac. erythraea* DNA from an *Xho*I site to a *Bam*HI site in the
35   *eryAII* gene of the erythromycin PKS cluster. These sites correspond to bases 23211 and 31581, respectively, of GenBank accession number M63676. The EryAT4 5'-flanking DNA

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was isolated by digesting pAIBX85 with *Msc*I and *Bst*EII (corresponding to nucleotides 23,211 and 31,581, respectively). The resulting 1800 bp DNA fragment was treated with the Klenow Fragment of DNA Polymerase I, ligated into the *Sma*I site of pUC19, and transformed into *E. coli* DH5α. Clones were selected on LB plates containing 150 μg/mL ampicillin and 50 μL of a 2% solution of X-gal for blue/white selection. The clones were confirmed by restriction analysis, resulting in the intermediate vector pUC/5'-flank. For convenient cloning of the NidAT5-encoding sequences, an *Avr*II site was engineered at the 3' end of the 5' flanking DNA. This was accomplished by PCR amplification from the *Pml*I site of the 5' flanking DNA to the *Bst*EII site with two oligonucleotides (SEQ ID NO:21 and SEQ ID NO:22). SEQ ID NO:22 incorporates an *Avr*II site and a *Bam*HI site at the 3' end of the 5' flanking DNA. PCR conditions were as described in EXAMPLE 20 using *Sac. erythraea* DNA as template with the following changes: Taq polymerase (GIBCO BRL) was used with the accompanying 10x buffer instead of Vent$_R$® DNA polymerase and cycling conditions were 96°C/30 sec, 55°C/30 sec, 72°C/30 sec for 25 cycles. The resulting 300 bp PCR fragment was then digested with *Pml*I and *Bam*HI, gel purified from a 1.0 % agarose gel with Prep-A-Gene, and ligated back into pUC/5'-flank digested with *Pml*I and *Bam*HI to give pUC/5'-flank-*Avr*II. The ligation was transformed into DH5α and plated onto LB plates containing 150 μg/mL ampicillin. Clones were confirmed by restriction analysis and DNA sequencing.

In order to clone the NidAT5-encoding DNA fragment downstream of the 5' flanking DNA, an *Avr*II site was also engineered at the 5' end of the NidAT5-encoding DNA. As depicted in FIG. 23, an *Avr*II site could be engineered into the NidAT5 DNA without altering the amino acid sequence. Two PCR oligonucleotides (SEQ ID NO:23 and SEQ ID NO:24) were designed to create an *Avr*II site at the 5' end and a *Bam*HI site at the 3' end, respectively, of the NidAT5-encoding DNA. A convenient *Fse*I site occurs naturally at the 3' end of NidAT5-encoding sequence, so the resulting PCR fragment contains an *Fse*I site just upstream of the PCR engineered *Bam*HI site. SEQ ID NO:23 and SEQ ID NO:24 were used in a PCR reaction with the template p16-2.2. This plasmid is pUC19 containing a 2.2 kb *Sma*I fragment from module 5 of the niddamycin PKS cluster (see FIG. 19), which encompasses the sequences encoding NidAT5. The resulting 1.0 kb PCR fragment was digested with *Avr*II and *Bam*HI, purified from a 1.0 % agarose gel using Prep-A-Gene, and cloned into the *Avr*II/*Bam*HI sites of pUC/5'-flank-*Avr*II. Clones were confirmed by restriction analysis and DNA sequencing, creating the intermediate plasmid pUC/5'-flank/ethAT.

The EryAT4 3'-flanking DNA was subcloned by digesting pAIBX85 with *Pml*I and *Msc*I, corresponding to nucleotides 29,231 and 31,209, respectively, from the *eryAII* gene

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(GenBank accession number M63676). The DNA was gel purified on a 1.0 % agarose gel using Prep-A-Gene and ligated into the *Sma*I site of pUC19. The ligation was transformed into DH5α and plated as described previously. Clones were confirmed by restriction analysis, resulting in the plasmid pUC/3'-flank.

5   Attachment of the EryAT4 3'-flanking DNA to the NidAT5 -encoding sequence was accomplished by digesting plasmid pUC/3'-flank with *Fse*I and *Bam*HI, gel purifying the fragment from a 1.0 % agarose gel using Prep-A-Gene, and ligating it into pUC/5'-flank/ethAT that had been previously digested with *Fse*I and *Bam*HI. The ligation was transformed into DH5α as before and clones were analyzed by restriction analysis, resulting

10   in the intermediate plasmid pUC/ethAT/C-6. The final step was to remove the NidAT5/flanking DNA insert from pUC/ethAT/C-6 with *Eco*RI and *Hind*III and ligate it into the *Eco*RI/*Hind*III sites of pCS5, resulting in the gene replacement/integration plasmid pEAT4 (FIG. 24).

15   EXAMPLE 22: Construction of *Sac. erythraea* ER720 EAT4-46

        An example of a 6-desmethyl-6-ethylerythromycin A producing microorganism was prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase domain in module 4 of the erythromycin PKS (EryAT4) of *Sac. erythraea* ER720 with a newly discovered DNA fragment encoding an ethylmalonyl acyltransferase domain (NidAT5) from

20   *S. caelestis* NRRL-2821. This was accomplished using the recombinant plasmid pEAT4, prepared as described in EXAMPLE 21. Transformation of *Sac. erythraea* ER720 and selection and confirmation of stable were carried out essentially as described in EXAMPLE 4. Nine thiostrepton sensitive colonies were selected and their chromosomal DNA cut with *Mlu*I and analyzed by Southern hybridization. In three of the nine thiostrepton sensitive

25   colonies, a probe consisting of an approximately 900 bp fragment spanning a KS/AT domain in *Streptomyces caelestis* hybridized with a chromosomal fragment of approximately 1.8 kb, indicating that NidAT5 had replaced EryAT4 in the chromosomes of these resolvants. The strain was named *Sac. erythraea* ER720 EAT4-46, referred to as simply EAT4-46.

30   EXAMPLE 23: Analysis of compounds produced by EAT4-46

        Compounds produced by strain EAT4-46, whose construction is described in EXAMPLE 22, were characterized by TLC, bioautography and mass spectrometry.

        The cells were grown in 30 mL of SCM for 4-5 days at 30°C. The culture was processed for TLC essentially as described in EXAMPLE 5. The results showed that EAT4-

35   46 produced a compound that migrated with the same rf as erythromycin A produced by wild type *Sac. erythraea* ER720, except in much lower yield.

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To determine the molecular mass of the compound, an ethyl acetate extract was prepared from a 50 mL SCM culture of EAT4-46 as described above, using a proportionate amount of reagents. The resulting residue was taken up in 50 μL of ethyl acetate and run on a TLC plate as described previously, except that the plate was not sprayed with anisaldehyde.

5   The compound of interest was isolated by scraping the silica resin in the vicinity of the spot and extracting the resin as described in EXAMPLE 8. Mass spectrometric analysis revealed that the compound produced by the EAT4-46 strain had a mass of 734, which corresponds to the molecular ion plus a proton (M+H$^+$) of erythromycin A.

In an attempt to increase substrate pools for the NidAT5 ethylmalonyl AT

10   construction, the EAT4-46 strain was grown in 100 mL of SCM media containing 50 mM butyric acid, pH 7.0. The culture was grown for 4 days at 30°C and then centrifuged for 10 minutes in a Sorval GLC-4 Centrifuge to pellet the cells. The resulting supernatant was adjusted to pH 9.0 by the addition of 600 μL of NH4OH and extracted twice with 1/2 volumes of ethyl acetate as described previously. After drying in a Speed-Vac rotary

15   concentrator, the extracted material was taken up in 100 μl of ethyl acetate and 10 μl was used for TLC analysis as described previously. Two spots running near eryA were observed in the butyric acid fed culture as opposed to only one spot in SCM media alone. To determine the molecular mass of the two spots, most of the remainder of the extract was again subjected to TLC, and the compounds in the eryA region of the plate were isolated as

20   described previously. Mass spectrometric analysis revealed that the two spots had molecular masses of 734 and 748. A molecular mass of 734 corresponds to the molecular ion plus a proton (M+H$^+$) of erythromycin A, whereas the species of molecular mass 748 is consistent with the molecular mass plus a proton (M+H$^+$) of ethylerythromycin A.

25   EXAMPLE 24: Cloning of the NidAT6 Domain from
Streptomyces caelestis NRRL-2821

A genomic library of _Streptomyces caelestis_ NRRL-2821 DNA was generated and screened with a probe specific for PKS genes as described in EXAMPLE 20. From Southern analysis of _Sst_I digests of the positive clones, some clones were selected for further analysis.

30   These clones were digested with _Sma_I and run on a 1% agarose gel for Southern hybridization with the PKS specific probe. The analysis revealed that a second cosmid, pCEL13f5, shared many hybridizing bands with pCEL18h5, but also contained two unique bands of 1.9 kb and 6.0 kb. This cosmid was chosen for further analysis in order to determine the sequence of the remaining PKS genes in the niddamycin pathway. Cosmid pCEL13f5

35   was digested with _Sst_I and the fragments were ligated to pUC19. A large _Sst_I fragment (>10 kb) was further digested with _Sma_I and ligated to pUC19. The ligations were transformed

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into DH5α cells and clones were selected on LB plates containing 150 μg/mL ampicillin and 50 μl of a 2% solution of X-gal for blue/white selection. DNA from clones containing the appropriate insert was isolated using the QIAprep Spin Plasmid Kit (QIAGEN Inc., Chatsworth, CA). Subclones were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), and the reactions were run on a 4.75% acrylamide, 8.3 M urea gel in an Applied Biosystems 373 DNA Sequencing System. Ordering of the inserts and motif identification was done as described in EXAMPLE 20.

The insert in cosmid pCEL13f5 was found to be approximately 25 kb in length, and the 5' end of the insert had about 10 kb of identical sequence with the 3' end of the insert in pCEL18h5. Together, the two cosmids contain all of the PKS genes of the niddamycin pathway (FIG. 19). Based on the structure of niddamycin (FIG. 20), the AT contained in module 6 (NidAT6) may utilize hydroxymalonate (tartronate) in the biosynthesis of the C-3, C-4, and O-4 positions of the macrolactone ring of niddamycin. (S. Omura *et al.* (*J. Antibiotics* 36:611-613 (1983)) have suggested that glycolate may be incorporated in the biosynthesis of the C-3, C-4 and O-4 positions of leucomycin, a closely related 16-membered macrolide). The nucleotide sequence of NidAT6 (top strand, SEQ ID NO:30) and its corresponding amino acid sequence (lower strand, SEQ ID NO:34) are shown in FIG. 25. A comparison of the amino acid sequence of NidAT6 with other ATs in the Swissprot database shows that NidAT6 resembles methylmalonyl ATs.

EXAMPLE 25: Construction of plasmid pUC18/NidAT6

Two PCR oligonucleotides (SEQ ID NO:25 and SEQ ID NO:26) are designed to subclone the 1024 bp DNA fragment encoding the NidAT6 domain from the niddamycin PKS cluster and to introduce two unique restriction sites, *Avr*II and *Nsi*I, for cassette cloning. This necessitates nucleotide changes, shown in bold in FIG. 26, at the beginning and near the end of the NidAT6-encoding DNA sequence. The changes shown also cause the replacement of a proline codon near the N-terminus of the NidAT6 domain with a valine codon, in order to increase the similarity of the domain junction sequence to that found naturally for some of the AT domains of the rapamycin PKS. (In FIG. 26, the underlined nucleotides are the wild-type sequence.) In addition, two other restriction sites, *Eco*RI and *Bgl*II, are also introduced at the 5' ends of the N-terminal and C-terminal oligonucleotides, respectively, for convenient subcloning of the PCR-generated product. The approximately 1 kb NidAT6 domain encoding DNA is amplified using methods described in Reagents and General Methods from Cosmid pCEL13f5. The PCR product is digested with *Eco*RI and *Bgl*II and subcloned into the *Eco*RI and *Bam*HI sites of pUC18. The ligation mixture is transformed into *E. coli* DH5α (GIBCO BRL) according to the manufacturer's instructions and transformants are selected on

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LB plates containing 150 µg/mL ampicillin and 50 µL of a 2% solution of X-gal for blue/white selection. Clones are confirmed by restriction analysis and the fidelity of the insert is confirmed by DNA sequencing. The final plasmid construct is named pUC18/NidAT6.

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EXAMPLE 26: Construction of plasmid pEryAT2/NidAT6

pEryAT2/NidAT6 is constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 26 and 27. To make a gene-replacement-vector specific for the eryAT2 domain, the two DNA regions immediately adjacent to eryAT2 are cloned and positioned adjacent to the DNA encoding the NidAT6 domain in order to allow homologous recombination to occur. The strategy and protocol for constructing the intermediate plasmid containing the flanking regions, pCS5/AT2-flank, are described in EXAMPLE 6 and FIG. 11. The final step in the construction of pEryAT2/NidAT6 is to ligate the 1 kb NidAT6-encoding DNA fragment having *Avr*II and *Nsi*I ends to pCS5/AT2-flank (EXAMPLE 6) cut with the same enzymes to give the gene replacement/integration plasmid pEryAT2/NidAT6 (FIG. 27). All ligation mixes are transformed into the intermediate host *E. coli* DH5α and clones are selected and characterized as described previously.

EXAMPLE 27: Construction of *Sac. erythraea* ER720 EryAT2/NidAT6

A 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-desmethyl-10-hydroxyerythromycin A producing microorganism is prepared by replacing the DNA fragment encoding the methylmalonyl acyltransferase domain of module 2 of the erythromycin PKS (EryAT2) of *Sac. erythraea* ER720 with a DNA fragment encoding a hydroxymalonyl acyltransferase domain (NidAT6) from *S. caelestis* NRRL-2821. This is accomplished with the recombinant plasmid, pEryAT2/NidAT6, prepared as described in EXAMPLE 26. Transformation of ER720 and selection and confirmation of stable resolvants are carried out essentially as described in EXAMPLE 4. Thiostrepton sensitive colonies are then selected and these are confirmed by Southern hybridization, using conditions described above, to have the EryAT2 replaced by NidAT6. The strain is designated *Sac. erythraea* ER720 EryAT2/NidAT6.

EXAMPLE 28: Analysis of compounds produced by
Sac. erythraea ER720 EryAT2/NidAT6

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Compounds produced by the recombinant *Sac. erythraea* strain, ER720
EryAT2/NidAT6, whose construction is described in EXAMPLE 27, are characterized by
TLC, bioassay, and mass spectrometry.

For TLC analysis cells are grown in either SGGP or SCM medium for 4-5 days at
30°C. The culture is processed for TLC essentially as described in EXAMPLE 5. Two novel
compounds predicted to be 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-
desmethyl-10-hydroxyerythromycin A, are expected to appear as blue spots running slightly
slower than erythromycin A.

To determine whether the novel spots seen on TLC have the molecular mass
corresponding to the predicted 10-desmethyl-10-hydroxyerythromycin A and 12-deoxy-10-
desmethyl-10-hydroxyerythromycin A, the remaining extract is further analyzed by mass
spectrometry. The two novel compounds are predicted to have masses of 736 and 720, which
correspond to the molecular ion plus a proton (M+H$^+$) of 10-desmethyl-10-
hydroxyerythromycin A and 12-deoxy-10-desmethyl-10-hydroxyerythromycin A,
respectively.

EXAMPLE 29: Construction of plasmid pEryATs/NidAT6

pEryATs/NidAT6 was constructed using standard methods of recombinant DNA
technology according to the schematic outlines of FIGS. 28 and 29. To construct a gene-
replacement vector specific for the eryATs domain, the two DNA regions immediately
adjacent to eryATs-encoding DNA were cloned and positioned adjacent to the NidAT6-
encoding DNA (EXAMPLE 25). The 5' and 3' boundaries of eryATs were designated as
nucleotides 902 and 1908, and correspond to the deposited *eryAI* sequence (GenBank
accession number M63676). To subclone the DNA fragment upstream of the eryATs domain
encoding region from the *Sac. erythraea* chromosome, two PCR oligonucleotides (SEQ ID
NO: 35 and SEQ ID NO: 36) were designed so that an *Eco*RI site was added at the 5' end of
the region and *Avr*II-*Bam*HI restriction sites were introduced at the 3' end. The 5'-flanking
region (about 1.2 kb) was generated by PCR using plasmid pAIEN22 DNA as template under
conditions described in EXAMPLE 2. The PCR product was subcloned into *Eco*RI and
*Bam*HI sites of pUC18 and the ligated DNA transformed into *E. coli* DH5α (GIBCO BRL)
according to the manufacturer's instructions. Clones were selected on LB plates containing
150 μg/mL ampicillin and 50 μL of a 2% solution of X-gal for blue/white selection. Clones
were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA
sequencing. The resulting construct was named pUC18/ATs/5'-flank.

For subcloning the 3'-flanking region of the eryATs from the *Sac. erythraea*
chromosome, two PCR oligonucleotides (SEQ ID NO: 37 and SEQ ID NO: 38 were designed
so that *Bam*HI-*Nsi*I restriction sites were introduced into the 5' end of the region and a

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*Hind*III restriction site was added to the 3' end. The 3'-flanking region (about 1.2 kb) was also generated by PCR using pAIEN22 as template as described above. The PCR fragment was subcloned into the *Bam*HI and *Hind*III sites of pUC18 and the ligated DNA transformed into *E. coli* DH5α as above. Clones were selected on LB plates containing 150 μg/mL

5   ampicillin and 50 μL of a 2% solution of X-gal for blue/white selection. Clones were confirmed by restriction analysis and the fidelity of the insert was confirmed by DNA sequencing. This intermediate construct was named pUC18/ATs/3'-flank (FIG. 28). The 1.2 kb *Eco*RI/*Bam*HI 5'-flanking fragment was isolated from pUC18/ATs/5'-flank and subcloned into the plasmid pCS5 cut with the same enzymes, generating pCS5/ATs/5'-flank. The 1.2 kb

10 *Bam*HI and *Hind*III 3'-flanking fragment was isolated from pUC18/ATs/3'-flank and then cloned into the pCS5/ATs/5'-flank vector cut with the same enzymes, resulting in pCS5/ATs-flank. The final step in the construction of pEryATs/NidAT6 was to ligate the 1 kb NidAT6 fragment having *Avr*II and *Nsi*I ends, isolated from pUC18/NidAT6 (EXAMPLE 25) to pCS5/ATs-flank cut with the same enzymes to give the gene replacement/integration plasmid

15 pEryATs/NidAT6 (FIG. 29). All ligation mixtures were transformed into the intermediate host *E. coli* DH5α and clones selected as previously described.

EXAMPLE 30: Construction of *Sac. erythraea* HATS

20 A 14-hydroxyerythromycin A producing microorganism was prepared by replacing the DNA fragment encoding the acyltransferase domain at the first AT of the amino terminus of the erythromycin PKS (EryATs) of *Sac. erythraea* ER720 that directs the loading of the starter unit propionyl CoA, with DNA encoding an hydroxymalonyl acyltransferase domain from the sixth module of the niddamycin PKS of *S. caelestis* NRRL-2821 (NidAT6). This

25 was accomplished with the recombinant plasmid, pEryATs/NidAT6, prepared as described in EXAMPLE 29. Transformation of ER720 and selection and confirmation of stable resolvants were carried out essentially as described in EXAMPLE 4. DNA isolated from thiostrepton-sensitive colonies were employed in Southern hybridizations, using stringency conditions described above, to identify clones that

30 had the EryATs domain replaced by NidAT6. The strain carrying such a replacement was designated *Sac. erythraea* HATS.

EXAMPLE 31: Analysis of compounds produced by *Sac. erythraea* HATS

35 *Sac. erythraea* HATS was grown in 30 mL of DOM medium (15.0 g soluble starch, 22.0 g soy flour, 2.0 g CaCO3, 1.5 g brewers yeast, 1.0 g MgSO4·7H2O, FeSO4·7H2O, 50

mL soybean oil per liter of distilled H2O) supplemented with 0.5% glycine for 7 days at 30°C and then centrifuged for 10 minutes in a clinical centrifuge to remove cells. The supernatant was removed to another tube and the pH adjusted to 9.0 by the addition of NH4OH. The supernatant was extracted twice with 15.0 mL of dichloromethane and the lower organic

5   phases were pooled and dried down. The residue was partitioned in a 10:10:1 mixture of heptane:methanol:0.05M KH2PO4 and the lower methanol/0.05M KH2PO4 layer was collected. A fresh 10:1 methanol/0.05M KH2PO4 mixture was added to the heptane phase and partitioned again. The methanol/0.05M KH2PO4 phases were pooled and dried down to remove the methanol, and the remaining aqueous phase was adjusted to pH 8 with 0.05M

10   KH2PO4, pH 8. This was then extracted twice with 1/2 volume of dichloromethane, the lower organic phases pooled, and then dried down. The residue was taken up in 30 μL of ethyl acetate and TLC performed essentially as described in EXAMPLE 5. A compound predicted to be erythromycin A was detected as a blue spot, along with a compound predicted to be 14-hydroxyerythromycin A, which also appeared as a blue spot but running slightly

15   below the erythromycin A spot.

To determine the mass of the compounds produced by *Sac. erythraea* HATS, a 16 μL sample from above of the extract was analyzed by mass spectrometry. The analysis identified a compound with mass 734, corresponding to the molecular ion plus a proton (M+H$^+$)of erythromycin A, as well as a compound with mass 736, which is consistent with the

20   molecular ion plus a proton (M+H$^+$) of 14-hydroxyerythromycin A.

EXAMPLE 32: Construction of plasmid pEryM1/NidAT6

25   pEryM1/NidAT6 was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIGS. 9 and 30. To construct a gene-replacement vector specific for the eryAT1 domain, the two DNA regions immediately adjacent to eryAT1 encoding DNA were cloned and positioned adjacent to the DNA encoding the NidAT6 domain in order to allow homologous recombination to occur. The

30   strategy and protocol for construction of the intermediate plasmid containing the eryAT1 flanking regions, pCS5/AT1-flank, are described in EXAMPLE 2 and FIG. 9 . The final step in the construction of pEryM1/nidAT6 was to first digest pUC18/NidAT6 (EXAMPLE 25) with AvrII and NsiI, and then ligate the 1 kb NidAT6 fragment generated into pCS5/AT1-flank cut with the same enzymes to give the gene-replacement/integration plasmid

35   pEryM1/NidAT6 (FIG. 30). All ligation mixes were transformed into the intermediate host *E. coli* DH5α and clones were selected and characterized as described previously.

EXAMPLE 33: Construction of *Sac. erythraea* HAT1

A 6-deoxy-12-desmethyl-12-epierythromycin A- and 12-desmethyl-12-
epierythromycin A-producing microorganism was prepared by replacing the DNA fragment
encoding the methylmalonyl acyltransferase domain of module 1 of the erythromycin PKS
(EryAT1) of *Sac. erythraea* ER720 with a DNA fragment encoding the hydroxymalonyl
acyltransferase domain (NidAT6) from *S. caelestis* NRRL-2821. This was accomplished
with the recombinant plasmid, pEryM1/NidAT6, prepared as described in EXAMPLE 32.
Transformation of *Sac. erythraea* ER720 and selection and confirmation of stable resolvants
were carried out essentially as described in EXAMPLE 4. DNA isolated from thiostrepton-
sensitive colonies were employed in Southern hybridizations, using stringency conditions
described above, to identify clones that had the EryATs domain replaced by NidAT6.The
strain carrying such a replacement was designated *Sac. erythraea* HAT1.

EXAMPLE 34: Analysis of compounds produced by
Sac. erythraea HAT1

Different compounds were produced by *Sac. erythraea* HAT1 (EXAMPLE 33)
depending upon the medium employed for growth. In one example, the culture was grown
for 5 days at 30°C in 50 mL SCM medium (20 g Soytone, 15 g Soluble Starch, 10.5 g MOPS,
1.5 g Yeast Extract and 0.1 g $CaCl_2$ per liter of distilled H_2O) supplemented with 10 mM
glycerol. The culture was then processed for TLC analysis essentially as described in
EXAMPLE 5. Compounds appearing as blue spots running in the region of erythromycin A
were detected. Mass spectrometry analysis of a 16 μL sample of the extract identified a
compound with mass 734, corresponding to the molecular ion plus a proton (M+H⁺) of
erythromycin A, as well as a compound with mass 704, which is consistent with the
molecular ion plus a proton (M+H⁺) of 6-deoxy-12-desmethyl-12-epierythromycin A.

In a second experiment, *Sac. erythraea* HATS1 was grown for 4 days at 30°C in 50
mL of the following medium: 15 g corn starch, 20 g soy flour, 1.5 g dried brewer's yeast, 10
g soybean oil, 1 g $CaCO_3$, 0.5 g $MgSO_4.7H_2O$, 0.015 g $FeSO_4$, and 1 g sodium pyruvate per
liter of distilled water. After growth, the culture was processed for TLC as described above.
Compounds appearing as blue spots running in the region of erythromycin A were detected.
Mass spectrometry analysis of a small sample of the extract identified a compound with mass
720, which is consistent with the molecular ion plus a proton (M+H⁺) of 12-desmethyl-12-
epierythromycin A.

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EXAMPLE 35: Cloning of rap ligase-PKS containing fragments from
Streptomyces hygroscopicus ATCC 29253

5   A 0.8 kb fragment encoding a segment of the *rapP* gene (Schwecke *et al.*, Proc. Natl.
Acad. Sci. 92, 7839-7843 [1995]) was amplified by PCR from genomic DNA prepared from
Streptomyces hygroscopicus ATCC 29253 using PCR conditions described in EXAMPLE 1
and primers SEQ ID NO: 39 and SEQ ID NO: 40. The resulting 0.8 kb DNA fragment was
labeled with ^{32}P using the Megaprime DNA labeling system (Amersham Life Science,

10   Arlington Heights, IL) and used as a probe to isolate cosmids containing *rapP* and adjacent
DNA in the following manner: a library of *Streptomyces hygroscopicus* ATCC 29253
genomic DNA (EXAMPLE 1) was transferred to LB agar plates to yield approximately 3,000
colonies/plate. After overnight growth the colonies were transferred from the plates to
Hybond-N nylon membranes (Amersham Life Science, Arlington Heights, IL), and probed

15   with the labeled *rapP* DNA segment following procedures outlined in Maniatis, *et al. supra.*
Hybridization was performed at 65 °C and a stringency wash was carried out with 0.1x SSC
at 65°C. Eleven positive cosmid clones were picked for restriction and PCR analysis. Five
cosmids were identified to contain both the *rapP* gene as well as adjacent DNA containing
segments of the *rapA* gene encoding a portion of the rapamycin PKS. The 5' end of the *rapA*

20   gene encodes the function referred to hereinafter as "rapligase" which is required for the
initiation of rapamycin biosynthesis. One of the cosmids, #2, was chosen as the DNA source
for PCR and subcloning to construct DNA containing rapligase and downstream PKS
domains for gene replacement.

25   EXAMPLE 36: Construction of plasmid pSL1180/rapligase 3.0

         Rapamycin biosynthesis is initiated by rapligase which employs the
dihydroxycyclohexyl-moiety of rapamycin as the starter. Adjacent to the rapligase domain is
the domain named ERS which is proposed to contain enoylreductase activity. A 3.0 kb

30   segment of *rapA* encoding the rapligase and ERS was inserted into plasmid pSL1180
(Brosius, J., *DNA* 8, 759, 1989) using standard methods of recombinant DNA technology, as
outlined in FIG. 31, to yield the plasmid pSL1180/rapligase 3.0. Since the rapligase-ERS-
containing segment was to be used to replace the starting segment of the erythromycin PKS,
thereby requiring the placement of the two unique restriction sites, *Avr*II and *Nsi*I, at the 5'-

35   and 3'-end of the 3.0 kb fragment, respectively, to facilitate subsequent cassette cloning, it

was necessary to assemble the 3.0 kb fragment containing rapligase and rapERS through a series of cloning steps shown in FIG. 31.

(a) Construction of pSL1180/0.11

PCR primers SEQ ID NO: 41 and SEQ ID NO: 42 were used to amplify a 0.11 kb N-terminal fragment of the rapligase domain using cosmid #2 (EXAMPLE 35) as the DNA template. Primer SEQ ID NO: 42 was designed to contain an *Avr*II site upstream of the sequence encoding the rapligase domain (FIG. 31). PCR reactions, cycling conditions and isolation of the desired fragment were as described in EXAMPLE 1. The PCR product was then cloned into *Eco*RI/*Sph*I sites of pSL1180, generating pSL1180/0.11(FIG. 31), and the sequence fidelity was confirmed by nucleotide sequencing.

(b) Construction of pSL1180/0.77

PCR primers SEQ ID NO: 43 and SEQ ID NO: 44 were used to amplify a 0.77 kb C-terminal fragment of the rapERS domain using cosmid #2 (EXAMPLE 35) as DNA template employing the conditions described immediately above. Primer SEQ ID NO:44 was designed to have a *Nsi*I site immediately downstream from the ERS domain. The PCR product was then cloned into *Xho*I/*Hind*III sites of pSL1180, generating pSL1180/0.77 (FIG. 31). The sequence fidelity was confirmed by nucleotide sequencing.

(c) Construction of pSL1180/0.11/2.1

A 2.1 kb *Sph*I/*Xho*I restriction fragment was isolated from cosmid #2 (EXAMPLE 35) and subcloned into the *Sph*I/*Xho*I sites of the pSL1180/0.11, generating pSL1180/0.11/2.1 (FIG. 31).

(d) Construction of pSL1180/rapligase 3.0.

A 0.77 kb *Xho*I/*Hind*III fragment was isolated from pSL1180/0.77 and subcloned into the *Xho*I/*Hind*III sites of the pSL1180/0.11/2.1, generating the plasmid pSL1180/rapligase 3.0 (FIG. 31).

EXAMPLE 37: Construction of plasmid pEryATs/rapligase 3.0

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Plasmid pEryATs/rapligase 3.0 was constructed using standard methods of recombinant DNA technology according to the schematic outlines of FIG. 32. The plasmid cassette which contains eryATs flanking regions, pCS5/ATs-flank, was constructed as described in EXAMPLE 29. As outlined in FIG. 32, a 3.0 kb *AvrII/NsiI* fragment isolated

5   from plasmid pSL1180/rapligase 3.0 (EXAMPLE 35), was subcloned into the same sites of pCS5/ATs-flank, generating gene replacement/integration plasmid pEryATs/rapligase 3.0 (FIG. 32).

EXAMPLE 38: Construction of *Sac. erythraea* EryATs/rapligase 3.0

10   An example of a 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A producing microorganism was prepared by replacing the DNA fragment encoding the acyltransferase domain at the amino terminus of the erythromycin PKS (EryATs) of *Sac. erythraea* ER720 with the DNA fragment encoding rapligase-rapERS domains (EXAMPLE 35) from *S. hygroscopicus* ATCC 29253. This was accomplished with the recombinant

15   plasmid, pEryATs/rapligase 3.0, prepared as described in EXAMPLE 37. Transformation of *Sac. erythraea* ER720 and selection and confirmation of stable resolvants were carried out as described in EXAMPLE 4. Four thiostrepton sensitive colonies were selected and one of them was confirmed by Southern hybridization to have the EryATs replaced by rapligase-rapERS. The strain was named *Sac. erythraea* EryATs/rapligase 3.0.

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EXAMPLE 39: Analysis of compounds produced by *Sac. erythraea* EryATs/rapligase 3.0

Sac. erythraea EryATs/rapligase 3.0, whose construction is described in EXAMPLE

25   38, was grown in 50 mL of SCM medium (EXAMPLE 34) for 2 days at 30°C, then supplemented each day with 1mM of 3,4-dihydroxycyclohexylcarboxylic acid for an additional 3 days. The culture then was processed for TLC analysis essentially as described in EXAMPLE 5. A novel compound predicted to be 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A, appeared as a blue spot running slightly slower

30   than erythromycin A.

To detect biological activity, a TLC-bioautography assay was performed essentially as described in EXAMPLE 5 but using 20 µL of the extracted sample. As with the positive controls, a small zone of inhibition developed around the sample spot indicating that the novel compound had bioactivity.

35   To determine whether the novel spot seen on TLC had the molecular mass corresponding to the predicted 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methyl-

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erythromycin A, a small sample of the ethylacetate extract was further analyzed by mass spectrometry. The mass spectrometric analysis revealed the novel compound to have a mass of 820, which corresponds to the predicted molecular ion plus a proton $(M+H^+)$ of 13-desethyl-13-(3',4'-dihydroxycyclohexyl)methylerythromycin A.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

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(v) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Diskette
   (B) COMPUTER: IBM Compatible
   (C) OPERATING SYSTEM: DOS
   (D) SOFTWARE: FastSEQ Version 2.0

(vi) CURRENT APPLICATION DATA:
   (A) APPLICATION NUMBER:
   (B) FILING DATE: 16-MAY-1979
   (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
   (A) APPLICATION NUMBER:
   (B) FILING DATE:

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        (2) INFORMATION FOR SEQ ID NO:1:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 925 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: double
         (D) TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
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CACGCCCAGA TCGCCCTGTT CGCCTTCGAA ACCGCCCTCT ACCGACTCTG GGAATCCTGG       240
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CAAAACCTGC CCCCGGCGG CGCCATGCTC GCCGTCGCCA CCGACCCCCA CACCCTCCAA       420
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CCCGTCCGCT ACACCGACAC CCTCCACCAC CTCCACCACC ACGGCATCAC CACCTACCTC       720
GAAATCGGCC CCGACACCAC CCTCACCGCC CTCGCCCGCA CCACCCTCCC CACCACCACC       780
CACCTCATCC CCACCACCCG CCGCAACCAC AACGAAGTCC GCAGCACGAA CGAGGCGTTG       840
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        (2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 1030 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: double
         (D) TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
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GCGCTCATCG ACCGTACGGT GTTCACACAG GCGGCTCTGT TCGCCGTGGA GACCTCCCTG       240
TTCCGGCTGT TCGAGGCCCA CGGCCTCGTC CCCGACTACC TCATCGGCCA CTCCATCGGC       300
GAAGTGACCG CGGCCCACCT GGCCGGGGTC CTCGATCTGG CGGACGCGTG CGTCCTGGTC       360
GCCCACCGCG GCCGCCTGAT GCAGTCGGCC CGGGCCGGCG GCGCGATGGC CGCGGTCCAG       420
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GGAGTCAACG GCCCGAACGC CACCGTCGTC TCCGGCGACG AGGACGCGGT CGAGCGGCTG       540
GTCGCGCGCT GGCGCGAGCA GGGCAGGCGG ACGAAGCGGC TGCCGGTCAG CCACGCCTTC       600
CACTCGCCGC ACATGGACGG GATCGTCGAC GAGTTCGTCA CCGCCGTCTC CGGGCTCACC       660
TTCCGCTCCC CGACGATCCC GGTCGTCTCC AACGTCACCG GGACCCTCGC CACCGTCGAC       720
CAGCTGACCT CGCCCGCGTA CTGGGCACGC CACATCCGCG AGGCCGTGCG CTTCGCCGAC       780
GGGGTGCGGT ACCTGGAGGG CGAGGGCGTC ACCGAATGGC TGGAGCTCGG GCCCGACGGC       840
```

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```
GTTCTCGTCG CCCTGGTCGA GGACTGCCTG GCGAAGGAGG CGGGATCGCT CGCGTCCGCC    900
CTGCGCAAGG GGGCGAGCGA GCCCCACACC GTGGGCGCGG CCATGGCCCG CGCGGTGCTG    960
CGCGGATCCG GCCCCGACTG GGCGGCGGTG TTCCCCGGCG CACGGCGGGT CGACCTTCCG   1020
ACGTATGCAT                                                          1030
```

(2) INFORMATION FOR SEQ ID NO:3:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 35 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATCTACACST CSGGCACSAC SGGCAAGCCS AAGGG                                35
```

(2) INFORMATION FOR SEQ ID NO:4:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 35 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
CTSAAGGCSG GCGGCGCSTA CGTSCCSATC GACCC                                35
```

(2) INFORMATION FOR SEQ ID NO:5:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 30 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CGCGAATTCC TAGGCTGGCG GTGATGTTCA                                      30
```

(2) INFORMATION FOR SEQ ID NO:6:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 33 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCGGATCCA TGCATACGTC GGCAGGGAGG TAC   33

     (2) INFORMATION FOR SEQ ID NO:7:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 28 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTCGAATTC GCTGGTCGCG GTGCACCT   28

     (2) INFORMATION FOR SEQ ID NO:8:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 32 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GACGGATCCG GCCCTAGGCT GCGCCCGGCT CG   32

     (2) INFORMATION FOR SEQ ID NO:9:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTGGGATCCT ATGCATTCCA GCGCGAGCGC   30

     (2) INFORMATION FOR SEQ ID NO:10:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 26 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

80

GAGAAGCTTG GCGCGACTTG CCCGCT   26

      (2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 36 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTTTTAAGC TTGGTACCTG CTCACCGGCA ACACCG   36

      (2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 42 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTTTTGGAT CCCTGCAGCC TAGGGTCGGA GGCACTGCCG GT   42

      (2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 37 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTTTCTGC AGTATGCATT CCAGGGCAAG CGGTTCT   37

      (2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 36 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTTTTGAAT TCACGCGTTG CCCGCGGCGT AGGCGC   36

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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 34 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCGAATTC CCTAGGACGG CAGTCCTGCT CACC   34

       (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 35 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATCGGATCC ATGCATACGT CGGAAGGTCG ACCCG   35

       (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 36 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTCGAAGAAT TCCCTAGGGT TGCCTTCCTG TTCGAC   36

       (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 36 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTCGAAAAGC TTATGCATAG ACCGGCAGAT CCACCG   36

       (2) INFORMATION FOR SEQ ID NO:19:

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```
        (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 19 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGTSAAGTC SAACATCGG                                                  19

            (2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 20 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCRATCTCRC CCTGCGARTG                                                 20

            (2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 44 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAGAGAGGAA CCAACGCGCA CGTGATCGTC GAAGAGGCAC CAGC                      44

            (2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 45 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAGAGAGGAT CCGACCTAGG CGCGGAGGTC ACCGGCGCGA CGGCG                     45

            (2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
```

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```
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear


(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GAGAGACCTA GGAAGCCGGT GTTCGTGTTC CCCGGCCAGG GCT                    43

    (2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 47 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear


(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAGAGAGGAT CCGAGGCCGG CCGTGCGCCC GGACCGAAGA CCGCCTC               47

    (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 41 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear


(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GAGAGAATTC CCTAGGGTCG CCTTCGTCTT TCCCGGGCAG G                     41

    (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 37 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear


(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTGAGATCTT ATGCATACGA GGGAAGCGGC ACCCTGC                          37

    (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 37 base pairs
```

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```
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTGAGATCTT ATGCATACGA GGGAAGCGGC ACCCTGC                                37


      (2) INFORMATION FOR SEQ ID NO:28:


      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 37 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTGAGATCTT ATGCATACGA GGGAAGCGGC ACCCTGC                                37


      (2) INFORMATION FOR SEQ ID NO:29:


      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 1010 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: double
         (D) TOPOLOGY: linear


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCCGACCGTG TCGTGTTCGT GTTCCCCGGC CAGGGCTCGC AGTGGGCCGG AATGGCCGAG        60
GGGCTGCTGG AGCGGTCCGG CGCGTTCCGG AGTGCGGCCG ACTCGTGCGA CGCCGCGCTG       120
CGGCCGTACC TCGGCTGGTC GGTGCTGAGC GTGCTGCGCG GGGAACCGGA CGCGCCCTCG       180
CTCGACCGGG TCGACGTCGT GCAGCCGGTG CTGTTCACGA TGATGGTCTC GCTCGCGGCG       240
GTCTGGCGTG CGCTGGGGGT GGAACCGGCG GCGGTCGTCG GCACTCGCA GGGTGAGATC        300
GCCGCTGCCC ATGTCGCCGG TGCGCTGTCG CTGGACGACT CGGCCCGGAT CGTCGCCCTG       360
CGCAGTCGGG CGTGGCTCGG ACTGGCGGGC AAGGGCGGCA TGGTGGCGGT GCCGATGCCG       420
GCGGAGGAGC TGCGGCCGCG GCTGGTGACG TGGGGGGACC GTCTGGCCGT CGCCGCCGTC       480
AACAGCCCCG GTTCCTGCGC CGTCGCAGGC GACCCGGAGG CGCTGGCCGA ACTGGTGGCG       540
CTGCTGACCG GTGAGGGGGT GCACGCCCGG CCGATCCCCG GCGTCGACAC GGCGGGCCAC       600
TCGCCGCAGG TGGACGCGTT GCGGGCTCAT CTGCTGGAGG TGCTGGCCCC GGTCGCCCCC       660
CGACCGGCCG ACATCCCGTT CTACTCGACG GTGACCGGCG GGCTGCTGGA CGGCACCGAG       720
CTGGACGCGA CGTACTGGTA CCGCAACATG CGCGAGCCCG TCGAGTTCGA GCGGGCCACA       780
CGGGCGCTGA TCGCCGACGG GCACGACGTC TTCCTGGAGA CGAGCCCGCA TCCCATGCTG       840
GCCGTGGCGC TGGAGCAGAC GGTCACCGAC GCCGGCACCG ACGCGGCGGT GCTCGGGACC       900
CTGCGCCGCC GCCACGGCGG TCCTCGCGCG CTGGCCCTGG CCGTCTGCCG CGCCTTCGCG       960
AGGCGGTCTT CGGTCCGGGC GCACGGCCCG TGGAGTTGCC CACCTATCCG                 1010


      (2) INFORMATION FOR SEQ ID NO:30:
```

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```
          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 1035 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: double
              (D) TOPOLOGY: linear


          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGCGCGCCTG CCTTCGTCTT TCCCGGGCAG GGCGCCCAGT GGGCCGGACT GGGAGCGCGG      60
CTCCTCGCGG ACTCCCCCGT CTTCCGCGCC AGGGCCGAGG CATGCGCGCG GGCGCTGGAG     120
CCTCACCTCG ACTGGTCGGT CCTCGACGTG CTGGCCGGCG CCCCGGGCAC CCCTCCCATC     180
GACCGGGCCG ACGTGGTGCA GCCGGTGCTG TTCACCACGA TGGTCTCGCT GGCCGCCCTC     240
TGGGAGGCCC ACGGGGTGCG GCCGGCCGCG GTCGTGGGCC ACTCCCAGGG CGAGGTGGCC     300
GCGGCCTGCG TGGCCGGTGC CCTGTCGCTG GACGACGCTG CCCTGGTGAT CGCCGGACGC     360
AGCAGGCTGT GGGGGCGGCT GGCCGGGAAC GGCGGGATGC TCGCGGTGAT GGCTCCGGCC     420
GAGCGGATCC GTGAGCTGCT CGAACCATGG CGGCAGCGGA TTTCGGTGGC GGCGGTCAAT     480
GGCCCCGCCT CGGTCACCGT CTCCGGTGAC GCGCTCGCGC TGGAGGAGTT CGGCGCGCGG     540
CTCTCCGCCG AGGGGGTGCT GCGCTGGCCG CTGCCGGGCG TCGACTTCGC CGGCCACTCG     600
CCGCAGGTGG AGGAGTTCCG CGCTGAGCTC CTGGACCTGC TCTCCGGCGT ACGGCCGGCT     660
CCTTCGCGGA TACCTTTCTT CTCCACCGTG ACGGCGGGTC CTTGCGGCGG CGACCAGCTG     720
GACGGGGCGT ACTGGTACCG CAACACGCGC GAACCCGTGG AGTTCGACGC CACGGTCCGG     780
GCGCTGCTGC GTGCGGGCCA TCACACGTTC ATCGAGGTCG GTCCGCATCC GCTGCTCAAC     840
GCCGCGATCG ACGAGATCGC AGCGGACGAG GGGGTAGCGG CCACGGCCCT GCATACGCTC     900
CAGCGGGGCG CTGGCGGCCT TGACCGCGTG CGCAACGCGG TGGGCGCCGC TTTCGCGCAC     960
GGTGTCCGGG TCGACTGGAA CGCCCTGTTC GAGGGCACCG GTGCGCGCAG GGTGCCGCTT    1020
CCCTCGTACG CCTTC                                                     1035

          (2) INFORMATION FOR SEQ ID NO:31:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 328 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear


          (ii) MOLECULE TYPE: None


          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Gly Pro Leu Ala Val Met Phe Thr Gly Gln Gly Ser Gln Arg Pro Gly
 1               5                  10                 15
Met Gly Arg Gln Leu Tyr Glu His Phe Pro Val Phe Ala Gln Ala Leu
            20                  25                 30
Asp Glu Val Phe Ala Leu Ala Thr Pro Gly Leu Arg Glu Val Met Phe
            35                  40                 45
Asp Pro Asp Gln Ala Glu Thr Leu Gln Arg Thr Asp His Ala Gln Ile
        50                  55                 60
Ala Leu Phe Ala Phe Glu Thr Ala Leu Tyr Arg Leu Trp Glu Ser Trp
65                  70                  75                 80
Gly Leu Arg Pro Asp Met Val Cys Gly His Ser Val Gly Glu Ile Thr
                85                  90                 95
Ala Ala His Val Ser Gly Thr Leu Thr Leu Pro Asp Ala Val His Leu
```

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```
                    100                  105                  110
Val Thr Thr Arg Gly Thr Leu Met Gln Asn Leu Pro Pro Gly Gly Ala
        115                  120                  125
Met Leu Ala Val Ala Thr Asp Pro His Thr Leu Gln Pro His Leu Asp
        130                  135                  140
Asn His His Asp Thr Ile Ser Ile Ala Ala Ile Asn Gly Pro His Ala
145                  150                  155                  160
Thr Val Leu Ser Gly Asp Arg Thr Thr Leu His His Ile Ala Thr Gln
                165                  170                  175
Leu Asn Thr Lys Thr Asn Trp Leu Asn Val Ser His Ala Phe His Ser
                180                  185                  190
Pro Leu Met Gln Pro Ile Leu Gln Pro Phe Thr Thr Thr Leu Asn Thr
        195                  200                  205
Leu Thr His His Pro Pro His Thr Pro Leu Ile Ser Met Leu Thr Ala
    210                  215                  220
Thr Pro Thr His Pro Asp Thr Thr His Trp Thr Gln His Ile Thr Ala
225                  230                  235                  240
Pro Val Arg Tyr Thr Asp Thr Leu His His Leu His His His Gly Ile
                245                  250                  255
Thr Thr Tyr Leu Glu Ile Gly Pro Asp Thr Thr Leu Thr Ala Leu Ala
                260                  265                  270
Arg Thr Thr Leu Pro Thr Thr Thr His Leu Ile Pro Thr Thr Arg Arg
        275                  280                  285
Asn His Asn Glu Val Arg Ser Thr Asn Glu Ala Leu Gly Arg Val Phe
        290                  295                  300
Ser Val Gly His Ser Val Asp Trp Arg Ala Leu Thr Pro Thr Gly Arg
305                  310                  315                  320
Arg Thr Ser Leu Pro Thr Tyr Pro
                325
```

        (2) INFORMATION FOR SEQ ID NO:32:

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 343 amino acids
                (B) TYPE: amino acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: None

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
Pro Arg Thr Ala Val Leu Leu Thr Gly Gln Gly Ser Gln Arg Gln Gly
1               5                   10                  15
Met Gly Arg Glu Leu Tyr Asp Arg Ser Pro Val Phe Ala Ala Ser Phe
        20                  25                  30
Asp Ala Ile Cys Ala Gln Leu Asp Gly Gln Leu Pro Arg Pro Leu Lys
        35                  40                  45
Asp Val Leu Phe Ala Pro Glu Gly Ser Glu Asp Ala Ala Leu Ile Asp
    50                  55                  60
Arg Thr Val Phe Thr Gln Ala Ala Leu Phe Ala Val Glu Thr Ser Leu
65                  70                  75                  80
Phe Arg Leu Phe Glu Ala His Gly Leu Val Pro Asp Tyr Leu Ile Gly
```

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```
                        85                    90                    95
His Ser Ile Gly Glu Val Thr Ala Ala His Leu Ala Gly Val Leu Asp
                    100                   105                   110
Leu Ala Asp Ala Cys Val Leu Val Ala His Arg Gly Arg Leu Met Gln
                115                   120                   125
Ser Ala Arg Ala Gly Gly Ala Met Ala Ala Val Gln Ala Ser Glu Asp
            130                   135                   140
Glu Val Arg Glu Ala Leu Ala Thr Phe Asp Asp Ala Val Ala Val Ala
145                   150                   155                   160
Gly Val Asn Gly Pro Asn Ala Thr Val Val Ser Gly Asp Glu Asp Ala
                165                   170                   175
Val Glu Arg Leu Val Ala Arg Trp Arg Glu Gln Gly Arg Arg Thr Lys
            180                   185                   190
Arg Leu Pro Val Ser His Ala Phe His Ser Pro His Met Asp Gly Ile
        195                   200                   205
Val Asp Glu Phe Val Thr Ala Val Ser Gly Leu Thr Phe Arg Ser Pro
    210                   215                   220
Thr Ile Pro Val Val Ser Asn Val Thr Gly Thr Leu Ala Thr Val Asp
225                   230                   235                   240
Gln Leu Thr Ser Pro Ala Tyr Trp Ala Arg His Ile Arg Glu Ala Val
                245                   250                   255
Arg Phe Ala Asp Gly Val Arg Tyr Leu Glu Gly Glu Gly Val Thr Glu
            260                   265                   270
Trp Leu Glu Leu Gly Pro Asp Gly Val Leu Val Ala Leu Val Glu Asp
        275                   280                   285
Cys Leu Ala Lys Glu Ala Gly Ser Leu Ala Ser Ala Leu Arg Lys Gly
    290                   295                   300
Ala Ser Glu Pro His Thr Val Gly Ala Ala Met Ala Arg Ala Val Leu
305                   310                   315                   320
Arg Gly Ser Gly Pro Asp Trp Ala Ala Val Phe Pro Gly Ala Arg Arg
                325                   330                   335
Val Asp Leu Pro Thr Tyr Ala
            340
```

(2) INFORMATION FOR SEQ ID NO:33:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 344 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Ala Asp Arg Val Val Phe Val Phe Pro Gly Gln Gly Ser Gln Trp Ala
1                   5                     10                    15
Gly Met Ala Glu Gly Leu Leu Glu Arg Ser Gly Ala Phe Arg Ser Ala
                20                    25                    30
Ala Asp Ser Cys Asp Ala Ala Leu Arg Pro Tyr Leu Gly Trp Ser Val
        35                    40                    45
Leu Ser Val Leu Arg Gly Glu Pro Asp Ala Pro Ser Leu Asp Arg Val
```

```
      50                         55                         60
Asp Val Val Gln Pro Val Leu Phe Thr Met Met Val Ser Leu Ala Ala
65                         70                         75                         80
Val Trp Arg Ala Leu Gly Val Glu Pro Ala Ala Val Val Gly His Ser
                      85                         90                         95
Gln Gly Glu Ile Ala Ala Ala His Val Ala Gly Ala Leu Ser Leu Asp
                      100                        105                        110
Asp Ser Ala Arg Ile Val Ala Leu Arg Ser Arg Ala Trp Leu Gly Leu
                 115                        120                        125
Ala Gly Lys Gly Gly Met Val Ala Val Pro Met Pro Ala Glu Glu Leu
            130                        135                        140
Arg Pro Arg Leu Val Thr Trp Gly Asp Arg Leu Ala Val Ala Ala Val
145                        150                        155                        160
Asn Ser Pro Gly Ser Cys Ala Val Ala Gly Asp Pro Glu Ala Leu Ala
                 165                        170                        175
Glu Leu Val Ala Leu Leu Thr Gly Glu Gly Val His Ala Arg Pro Ile
                 180                        185                        190
Pro Gly Val Asp Thr Ala Gly His Ser Pro Gln Val Asp Ala Leu Arg
            195                        200                        205
Ala His Leu Leu Glu Val Leu Ala Pro Val Ala Pro Arg Pro Ala Asp
       210                        215                        220
Ile Pro Phe Tyr Ser Thr Val Thr Gly Gly Leu Leu Asp Gly Thr Glu
225                        230                        235                        240
Leu Asp Ala Thr Tyr Trp Tyr Arg Asn Met Arg Glu Pro Val Glu Phe
                 245                        250                        255
Glu Arg Ala Thr Arg Ala Leu Ile Ala Asp Gly His Asp Val Phe Leu
                 260                        265                        270
Glu Thr Ser Pro His Pro Met Leu Ala Val Ala Leu Glu Gln Thr Val
            275                        280                        285
Thr Asp Ala Gly Thr Asp Ala Ala Val Leu Gly Thr Leu Arg Arg Arg
       290                        295                        300
His Gly Gly Pro Arg Ala Leu Ala Leu Ala Val Cys Arg Ala Phe Ala
305                        310                        315                        320
His Gly Val Glu Val Asp Pro Glu Ala Val Phe Gly Pro Gly Ala Arg
                 325                        330                        335
Pro Val Glu Leu Pro Thr Tyr Pro
            340
```

(2) INFORMATION FOR SEQ ID NO:34:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 345 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
Arg Ala Pro Ala Phe Val Phe Pro Gly Gln Gly Ala Gln Trp Ala Gly
1                   5                   10                  15
Leu Gly Ala Arg Leu Leu Ala Asp Ser Pro Val Phe Arg Ala Arg Ala
```

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```
                 20                  25                  30
Glu Ala Cys Ala Arg Ala Leu Glu Pro His Leu Asp Trp Ser Val Leu
            35                  40                  45
Asp Val Leu Ala Gly Ala Pro Gly Thr Pro Pro Ile Asp Arg Ala Asp
        50                  55                  60
Val Val Gln Pro Val Leu Phe Thr Thr Met Val Ser Leu Ala Ala Leu
65                  70                  75                  80
Trp Glu Ala His Gly Val Arg Pro Ala Ala Val Val Gly His Ser Gln
            85                  90                  95
Gly Glu Val Ala Ala Ala Cys Val Ala Gly Ala Leu Ser Leu Asp Asp
            100                 105                 110
Ala Ala Leu Val Ile Ala Gly Arg Ser Arg Leu Trp Gly Arg Leu Ala
        115                 120                 125
Gly Asn Gly Gly Met Leu Ala Val Met Ala Pro Ala Glu Arg Ile Arg
    130                 135                 140
Glu Leu Leu Glu Pro Trp Arg Gln Arg Ile Ser Val Ala Ala Val Asn
145                 150                 155                 160
Gly Pro Ala Ser Val Thr Val Ser Gly Asp Ala Leu Ala Leu Glu Glu
            165                 170                 175
Phe Gly Ala Arg Leu Ser Ala Glu Gly Val Leu Arg Trp Pro Leu Pro
            180                 185                 190
Gly Val Asp Phe Ala Gly His Ser Pro Gln Val Glu Glu Phe Arg Ala
            195                 200                 205
Glu Leu Leu Asp Leu Leu Ser Gly Val Arg Pro Ala Pro Ser Arg Ile
    210                 215                 220
Pro Phe Phe Ser Thr Val Thr Ala Gly Pro Cys Gly Gly Asp Gln Leu
225                 230                 235                 240
Asp Gly Ala Tyr Trp Tyr Arg Asn Thr Arg Glu Pro Val Glu Phe Asp
            245                 250                     255
Ala Thr Val Arg Ala Leu Leu Arg Ala Gly His His Thr Phe Ile Glu
            260                 265                 270
Val Gly Pro His Pro Leu Leu Asn Ala Ala Ile Asp Glu Ile Ala Ala
            275                 280                 285
Asp Glu Gly Val Ala Ala Thr Ala Leu His Thr Leu Gln Arg Gly Ala
    290                 295                 300
Gly Gly Leu Asp Arg Val Arg Asn Ala Val Gly Ala Ala Phe Ala His
305                 310                 315                 320
Gly Val Arg Val Asp Trp Asn Ala Leu Phe Glu Gly Thr Gly Ala Arg
            325                 330                 335
Arg Val Pro Leu Pro Ser Tyr Ala Phe
            340                 345
```

    (2) INFORMATION FOR SEQ ID NO:35:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 28 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: None

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

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TTTGAATTCA CGTCCTCGAC GTGCAGCA   28

        (2) INFORMATION FOR SEQ ID NO:36:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TTTGGATCCC CTAGGGGACG GCCGGGCCAC GCC   33

         (2) INFORMATION FOR SEQ ID NO:37:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TTTGGATCCA TGCATCTGCC GGAGTTCGCC CCG   33

        (2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTTAAGCTTG CGCCCGCCCG TTGGGC   26

        (2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

ATGGCTTCCG ACAGTCCCCG CCCAAGGCCG   30

        (2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

ACCAATTCCG TCGGCGGGCA CCAGGCCACC   30

        (2) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TTTTGAATTC CCTAGGATGT CACGCGCGGA ACTGG   35

        (2) INFORMATION FOR SEQ ID NO:42:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: None

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TTTTGCATGC GTCAGTGCGA GCCG   24

        (2) INFORMATION FOR SEQ ID NO:43:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

TTTTCTCGAG GTCGGCCCGG AAGT   24

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 36 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TTTTAAGCTT ATGCATGTCG AGTCGCCGGG GAATGG   36

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What is claimed is:

1.   A compound of the formula:
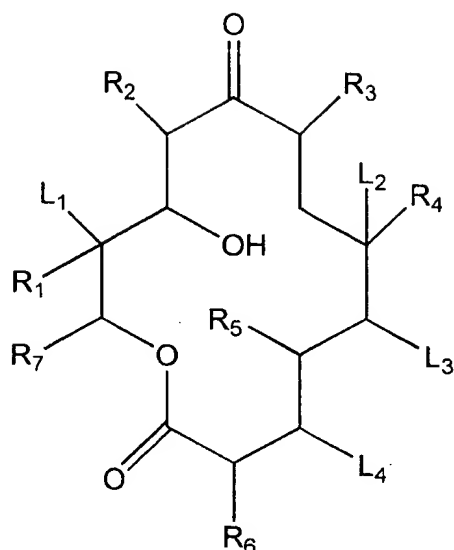
X

wherein

R_1, R_2, R_3, R_4, R_5, and R_6 are independently selected from Q wherein Q is selected from the group consisting of (a) -H, (b) -Me, (c) -Et, and (d) -OH;

R_7 is selected from the group consisting of -Et, -HOMe (hydroxymethyl), and 3,4-dihydroxycyclohexylmethyl;

L_1 and L_2 are independently -H or -OH;

L_3 is D-desosamine or -OH; and

L_4 is L-mycarose, L-cladinose or -OH
with the proviso that when R_7 is -Et and R_1-R_5 are -Me, R_6 is other than -H or -Me

2.   The compound of claim 1 wherein Q is selected from the group consisting of (a), (b), and (c), R_7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

3.   The compound of claim 1 wherein Q is selected from the group consisting of (a), (b), and (d), R_7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

4.   The compound of claim 1 wherein Q is selected from the group consisting of (a), (c), and (d), R_7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

5.   The compound of claim 1 wherein Q is selected from the group consisting of (b), (c), and (d), R_7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

6.   The compound of claim 1 wherein

        (a) R_6 and R_1 are -H and R_2, R_3, R_4 and R_5 are -Me,

        (b) R_5 and R_1 are -H and R_2, R_3, R_4 and R_6 are -Me,

        (c) R_4 and R_1 are -H and R_2, R_3, R_5 and R_6 are -Me,

        (d) R_3 and R_1 are -H and R_2, R_4, R_5 and R_6 are -Me,

        (e) R_2 and R_1 are -H and R_3, R_4, R_5 and R_6 are -Me,

        (f) R_6 and R_2 are -H and R_1, R_3, R_4 and R_5 are -Me,

        (g) R_5 and R_2 are -H and R_1, R_3, R_4 and R_6 are -Me,

        (h) R_4 and R_2 are -H and R_1, R_3, R_5 and R_6 are -Me,

        (i) R_3 and R_2 are -H and R_1, R_4, R_5 and R_6 are -Me,

        (j) R_6 and R_3 are -H and R_1, R_2, R_4 and R_5 are -Me,

        (k) R_5 and R_3 are -H and R_1, R_2, R_4 and R_6 are -Me,

        (l) R_4 and R_3 are -H and R_1, R_2, R_5 and R_6 are -Me,

        (m) R_6 and R_4 are -H and R_1, R_2, R_3 and R_5 are -Me,

        (n) R_5 and R_4 are -H and R_1, R_2, R_3 and R_6 are -Me, or

        (o) R_6 and R_5 are -H and R_1, R_2, R_3 and R_4 are -Me;

        R_7 is -Et; and L_1, L_2, L_3 and L_4 are as defined therein.

7.   The compound of claim 6 wherein (a)-(o) and R_7 are as defined therein, L_1 and L_2 are -OH, L_3 is D-desosamine and L_4 is L-cladinose.

8.   The compound of claim 1 wherein

        (a) R_6, R_2 and R_1 are -H and R_3, R_4 and R_5 are -Me,

        (b) R_5, R_2 and R_1 are -H and R_3, R_4 and R_6 are -Me,

        (c) R_4, R_2 and R_1 are -H and R_3, R_5 and R_6 are -Me,

        (d) R_3, R_2 and R_1 are -H and R_4, R_5 and R_6 are -Me,

        (e) R_6, R_3 and R_1 are -H and R_2, R_4 and R_5 are -Me,

        (f) R_5, R_3 and R_1 are -H and R_2, R_4 and R_6 are -Me,

        (g) R_4, R_3 and R_1 are -H and R_2, R_5 and R_6 are -Me,

        (h) R_6, R_4 and R_1 are -H and R_2, R_3 and R_5 are -Me,

        (i) R_5, R_4 and R_1 are -H and R_2, R_3 and R_6 are -Me,

        (j) R_6, R_5 and R_1 are -H and R_2, R_3 and R_4 are -Me,

        (k) R_6, R_3 and R_2 are -H and R_1, R_4 and R_5 are -Me,

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(l) R_5, R_3 and R_2 are -H and R_1, R_4 and R_6 are -Me,

(m) R_4, R_3 and R_2 are -H and R_1, R_5 and R_6 are -Me,

(n) R_6, R_4 and R_2 are -H and R_1, R_3 and R_5 are -Me,

(o) R_5, R_4 and R_2 are -H and R_1, R_3 and R_6 are -Me,

(p) R_6, R_5 and R_2 are -H and R_1, R_3 and R_4 are -Me,

(q) R_6, R_4 and R_3 are -H and R_1, R_2 and R_5 are -Me,

(r) R_5, R_4 and R_3 are -H and R_1, R_2 and R_6 are -Me,

(s) R_6, R_5 and R_3 are -H and R_1, R_2 and R_4 are -Me, or

(t) R_6, R_5 and R_4 are -H and R_1, R_2 and R_3 are -Me;

R7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

9.   The compound of claim 8 wherein (a)-(t) and R7 are as defined therein, L_1 and L_2 are -OH, L_3 is D-desosamine and L_4 is L-cladinose.

10.   The compound of claim 1 wherein

(a) R_6, R_3, R_2 and R_1 are -H and R_5, and R_4 are -Me,

(b) R_5, R_3, R_2 and R_1 are -H and R_6, and R_4 are -Me,

(c) R_4, R_3, R_2 and R_1 are -H and R_5, and R_6 are -Me,

(d) R_6, R_4, R_2 and R_1 are -H and R_3, and R_5 are -Me,

(e) R_5, R_4, R_2 and R_1 are -H and R_3, and R_6 are -Me,

(f) R_6, R_5, R_2 and R_1 are -H and R_3, and R_4 are -Me,

(g) R_6, R_4, R_3 and R_1 are -H and R_2, and R_5 are -Me,

(h) R_5, R_4, R_3 and R_1 are -H and R_2, and R_6 are -Me,

(i) R_6, R_5, R_4 and R_1 are -H and R_2, and R_3 are -Me,

(j) R_2, R_4, R_3 and R_1 are -H and R_5, and R_6 are -Me,

(k) R_6, R_4, R_3 and R_2 are -H and R_1, and R_5 are -Me,

(l) R_5, R_4, R_3 and R_2 are -H and R_1, and R_6 are -Me,

(m) R_6, R_5, R_3 and R_2 are -H and R_1, and R_4 are -Me, or

(n) R_6, R_5, R_4 and R_3 are -H and R_1, and R_2 are -Me;

R7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

11.   The compound of claim 10 wherein (a)-(n) and R7 are as defined therein, L_1 and L_2 are -OH, L_3 is D-desosamine and L_4 is L-cladinose.

12.   The compound of claim 1 wherein

(a) R_5, R_4, R_3, R_2 and R_1 are -H and R_6 is -Me,

(b) R_6, R_4, R_3, R_2 and R_1 are -H and R_5 is -Me,

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    (c) R_6, R_5, R_3, R_2 and R_1 are -H and R_4 is -Me,

    (d) R_6, R_5, R_4, R_2 and R_1 are -H and R_3 is -Me,

    (e) R_6, R_5, R_4, R_3 and R_1 are -H and R_2 is -Me, or

    (f) R_6, R_5, R_4, R_3 and R_2 are -H and R_1 is -Me;

R_7 is -Et and L_1, L_2, L_3 and L_4 are as defined therein.

13.   The compound of claim 12 wherein (a)-(f) and R_7 are as defined therein, L_1 and L_2 are -OH, L_3 is D-desosamine and L_4 is L-cladinose.

14.   The compound of claim 1 wherein R_1, R_2, R_3, R_4, R_5 and R_6 are -H and R_7, L_1, L_2, L_3 and L_4 are as defined therein.

15.   The compound of claim 14 wherein R_1, R_2, R_3, R_4, R_5, R_6 and R_7 are as defined therein, L_1 and L_2 are -OH, L_3 is D-desosamine and L_4 is L-cladinose.

16.   The compound of claim 1 selected from the group consisting of 6,10-didesmethyl-6-ethylerythromycin A; 10,12-didesmethyl-12-deoxy-12-ethylerythromycin A; 10,12-didesmethyl-12-deoxy-10-hydroxyerythromycin A; 6,10,12-tridesmethyl-6,12-diethylerythromycin A, and 6,10,12-tridesmethyl-6-deoxy-6,12-diethylerythromycin A.

17.   The compound of claim 1 selected from the group consisting of 10-desmethylerythronolide B, 10-desmethyl-6-deoxyerythronolide B, 12-desmethylerythronolide B, 12-desmethyl-6-deoxyerythronolide B, 12-desmethyl-12-ethylerythronolide B, 6-desmethyl-6-deoxy-6-ethylerythronolide B, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 10-desmethyl-6,12-dideoxyerythromycin A, 12-desmethylerythromycin A, 12-desmethyl-12-deoxyerythromycin A, 12-desmethyl-6,12-dideoxyerythromycin A, 6-desmethyl-6-ethylerythromycin A, 12-desmethyl-12-ethylerythromycin A, 12-desmethyl-12-deoxy-12-ethylerythromycin A, 10-desmethyl-10-hydroxyerythromycin A, 12-desmethyl-12-epihydroxyerythromycin A, 10,12-didesmethylerythromycin A, 10,12-didesmethyl-12-deoxyerythromycin A, and 10,12-didesmethyl-6,12-dideoxyerythromycin A.

18.   The compound of claim 1 selected from the group consisting of 10-desmethylerythronolide B, 10-desmethyl-6-deoxyerythronolide B, 12-desmethylerythronolide B, 12-desmethyl-6-deoxyerythronolide B, 10-desmethylerythromycin A, 10-desmethyl-12-deoxyerythromycin A, 10-desmethyl-6,12-dideoxyerythromycin A, 12-desmethylerythromycin A, 12-desmethyl-12-deoxyerythromycin A, 12-desmethyl-6,12-

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dideoxyerythromycin A, 10,12-didesmethylerythromycin A, 10,12-didesmethyl-12-
deoxyerythromycin A, and 10,12-didesmethyl-6,12-dideoxyerythromycin A.

19.   A compound selected from the group consisting of 10-desmethylerythromycin A, 10-
desmethyl-12-deoxyerythromycin A, and 12-desmethyl-12-deoxyerythromycin A.

20.   The compound of claim 1 selected from the group consisting of 8-desmethyl-8-
hydroxyerythromycin A, 6-desmethyl-6-epierythromycin A, 4-desmethyl-4-
hydroxyerythromycin A, 2-desmethyl-2-hydroxyerythromycin A and 13-desethyl-13-
hydroxymethol erythromycin A.

21.   The compound of claim 1 selected from the group consisting of 2,12-didesmethyl-
2,12-dihydroxyerythromycin, 4,10-didesmethyl-4,10-dihydroxyerythromycin, 10,12-
didesmethyl-10-hydroxyerythromycin, and 6,10-didesmethyl-6-ethyl-10-
hydroxyerythromycin A.

22.   The compound of claim 1 which is 13-desethyl-13-(3',4'-
dihydroxycyclohexyl)methylerythromycin A.

23.   An isolated polynucleotide sequence or fragment thereof which encodes an
enzymatically active acyltransferase domain from a polyketide-producing microorganism
selected from the group consisting of *Streptomyces hygroscopicus*, *Streptomyces venezuelae*,
and *Streptomyces caelestis*.

24.   The polynucleotide of Claim 23 selected from the group consisting of SEQ ID NO:1,
SEQ ID NO:2, SEQ ID NO:29 and SEQ ID NO:30.

25.   The polynucleotide of Claim 23 wherein said acyltransferase domain is selected from
the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33 and SEQ ID NO:34.

26.   A vector comprising a polynucleotide sequence or fragment thereof which encodes an
enzymatically active acyltransferase domain from *Streptomyces*.

27.   The vector of Claim 26 wherein said *Streptomyces* is selected from the group
consisting of *Streptomyces hygroscopicus*, *Streptomyces venezuelae*, and *Streptomyces
caelestis*.

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28.   The vector of Claim 26 wherein said polynucleotide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:29 and SEQ ID NO:30.

29.   The vector of Claim 26 wherein said acyltransferase domain is selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33 and SEQ ID NO:34.

30.   A vector selected from the group consisting of pUC18/LigAT2, pEryAT1/LigAT2, pEryAT2/LigAT2, pUC18/venAT, pEryAT1/venAT, pUC19/rapAT14, pEryAT1/rapAT14, pEryAT2/rapAT14, pUC/5'-flank/ethAT, pUC/ethAT/C-6, pEAT4, pUC18/NidAT6, pEryAT2/NidAT6, pEryATs/NidAT6, and pEryATs/rapligase 3.0..

31.   A host cell transformed with the vector of Claim 32.

32.   The host cell of Claim 31 wherein said cell is a bacterial cell or a polyketide-producing microorganism.

33.   The host cell of Claim 32 wherein said polyketide-producing microorganism is selected from the group consisting of *Saccharopolyspora* species and *Streptomyces* species.

34.   The host cell of Claim 33 wherein said polyketide-producing microorganism is *Saccharopolyspora erythraea*.

35.   A method for altering the substrate specificity of a polyketide synthase in a first polyketide-producing microorganism comprising the steps of:
      (a)   isolating a first and second genomic DNA segment, each comprising a polyketide synthase wherein said first genomic DNA segment is from said first polyketide-producing microorganism and said second genomic DNA segment is from said first polyketide-producing microorganism or a second polyketide-producing microorganism;
      (b)   identifying one or more discrete fragments of said first genomic DNA segment, each of which encodes an acyltransferase domain;
      (c)   identifying one or more discrete fragments of said second genomic DNA segment, each of which encodes a related domain to said acyltransferase domain of said first genomic DNA segment; and
      (d)   transforming a cell of said first polyketide-producing microorganism with one or more of said fragments from step (c) under conditions suitable for the occurrence of a homologous recombination event, leading to the replacement of one or more of said

fragments from said first genomic DNA segment with one or more of said fragments from step (c).

36.   The method of Claim 35 wherein said first polyketide-producing microorganism is *Saccharopolyspora erythraea*.

37.   The method of Claim 35 wherein said second polyketide-producing microorganism is *Streptomyces*.

38.   The method of Claim 35 wherein said second polyketide-producing microorganism is *Saccharopolyspora erythraea*.

39.   The method of Claim 35 wherein said related domain is selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33 and SEQ ID NO:34.
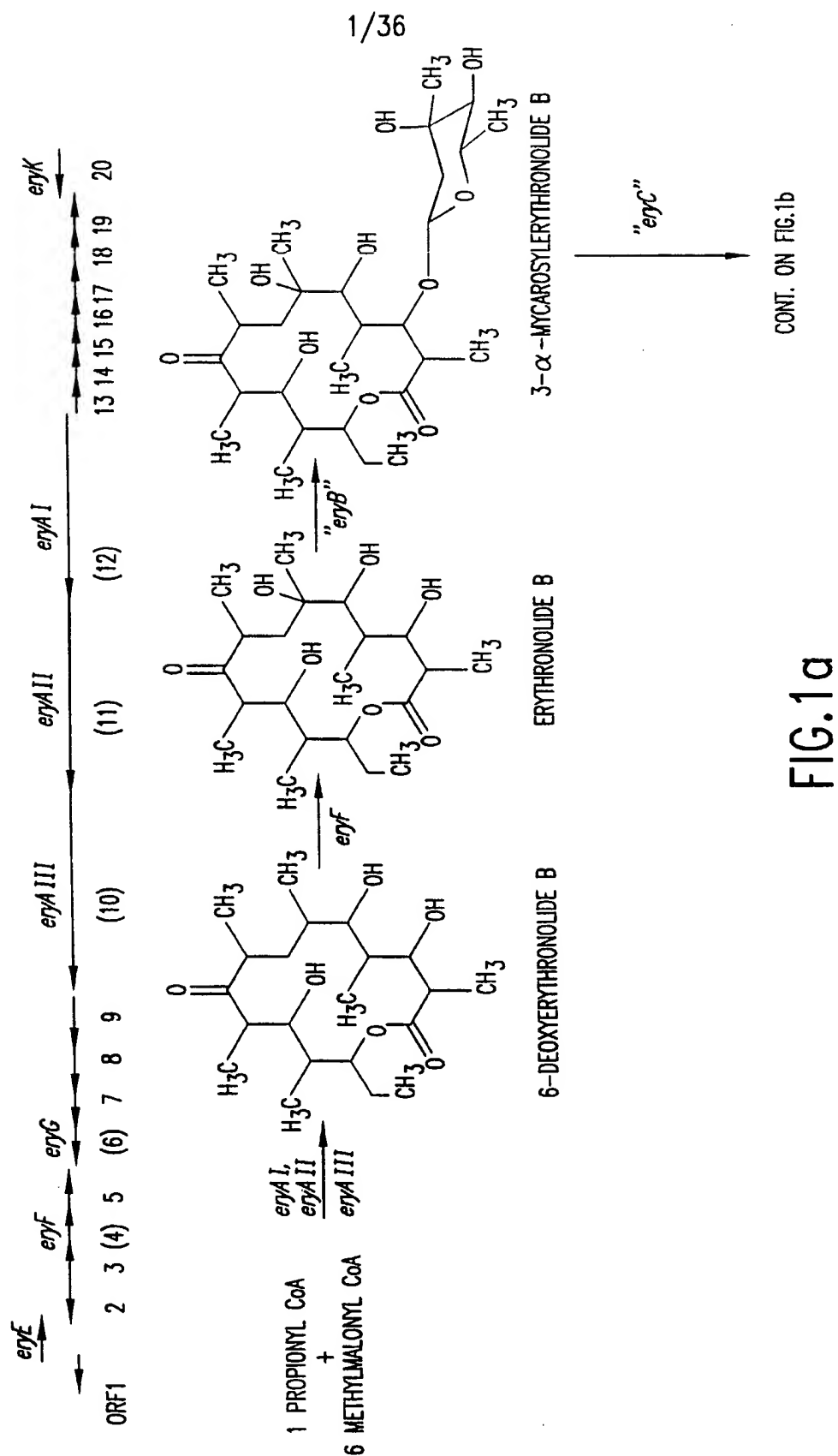
FIG.1a
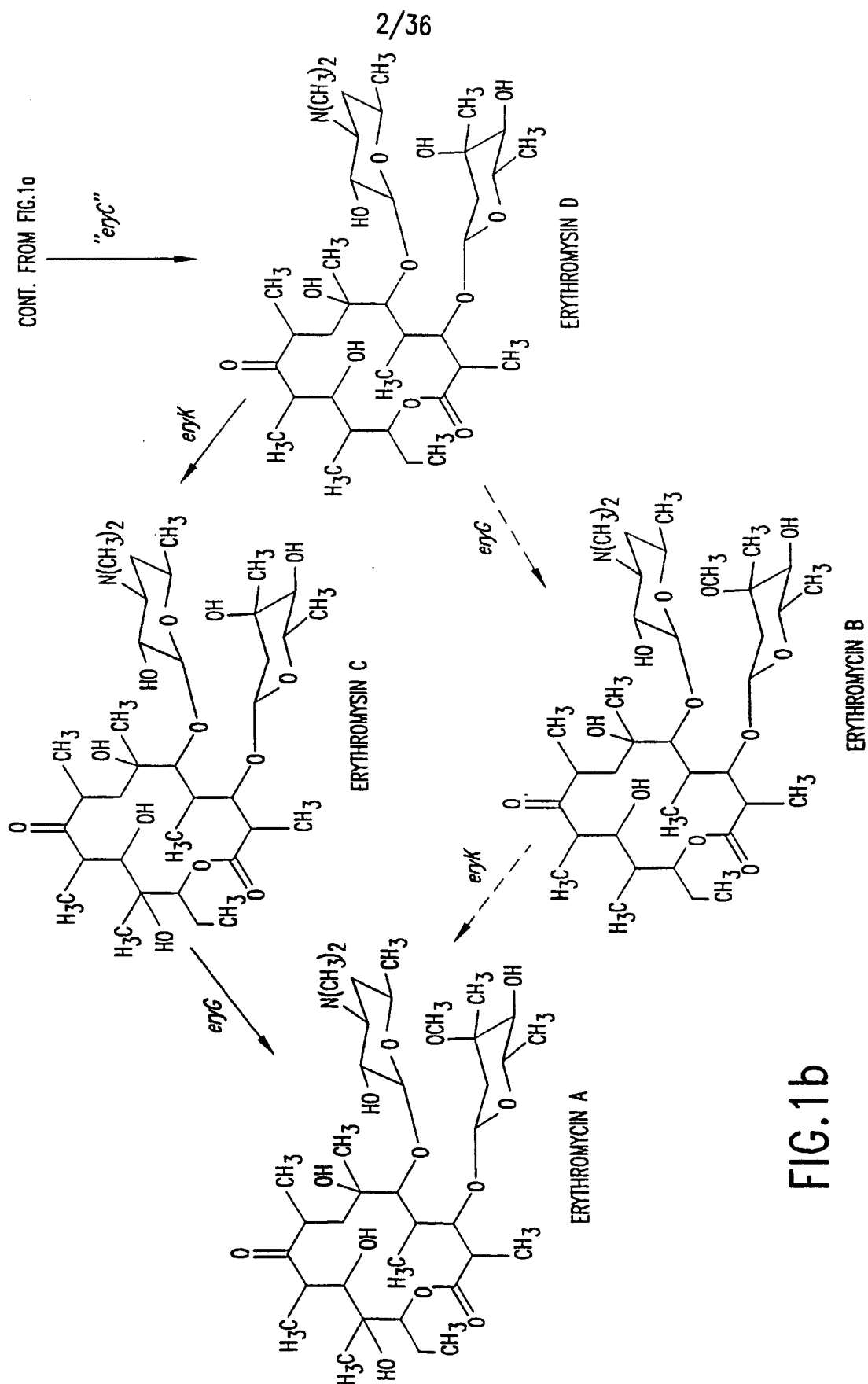
FIG.1b
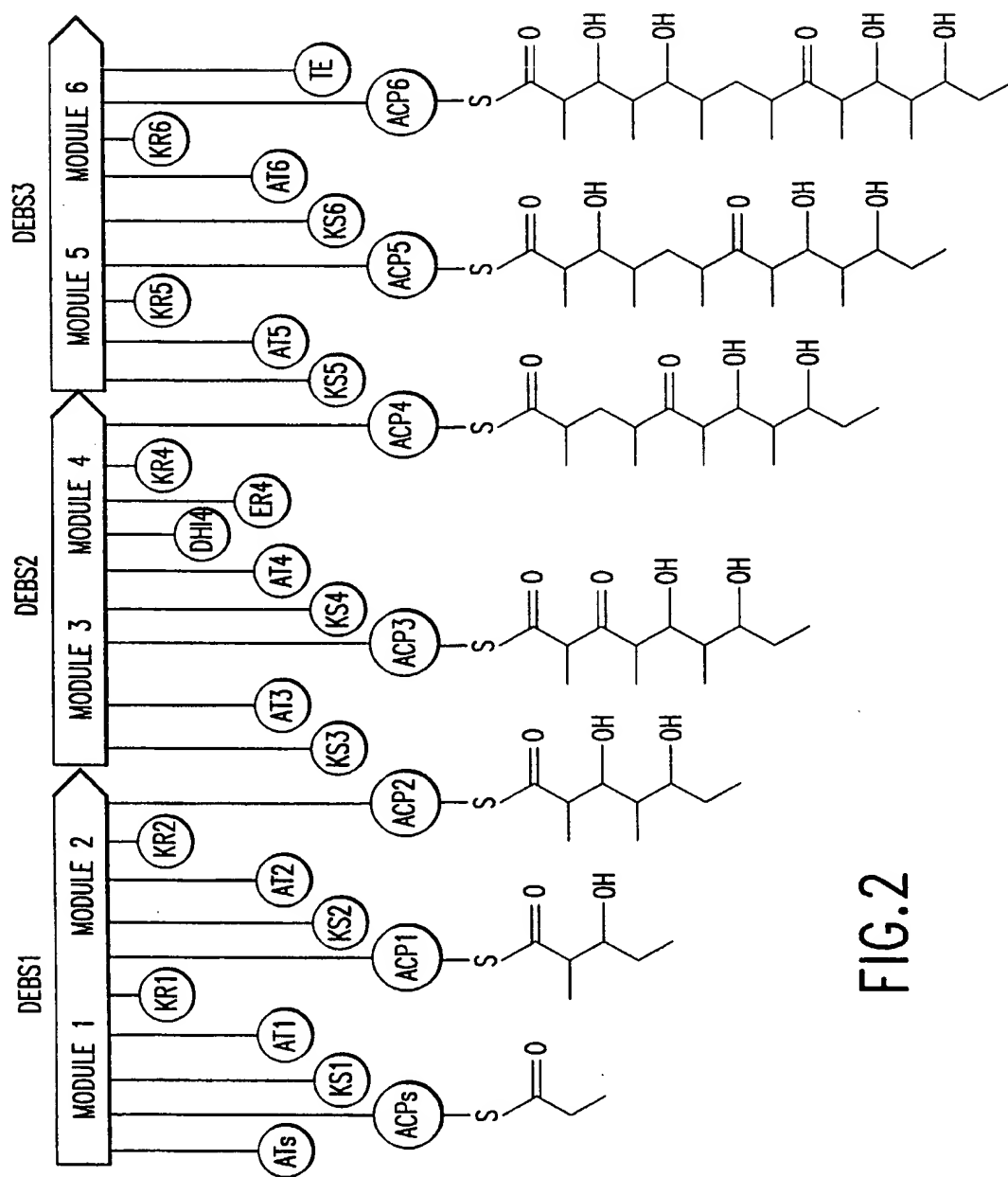
FIG.2

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FIG.3
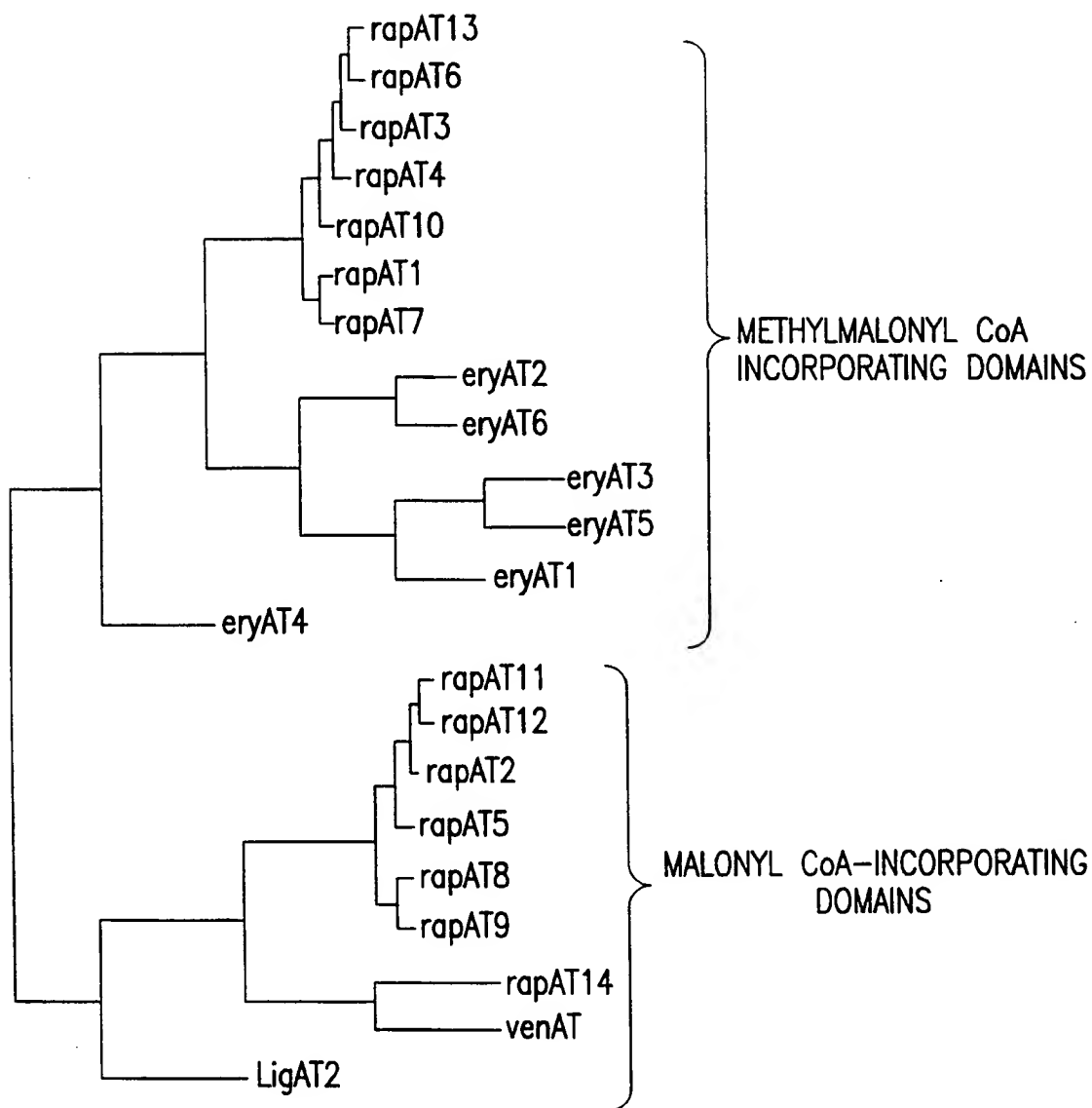
FIG.4a
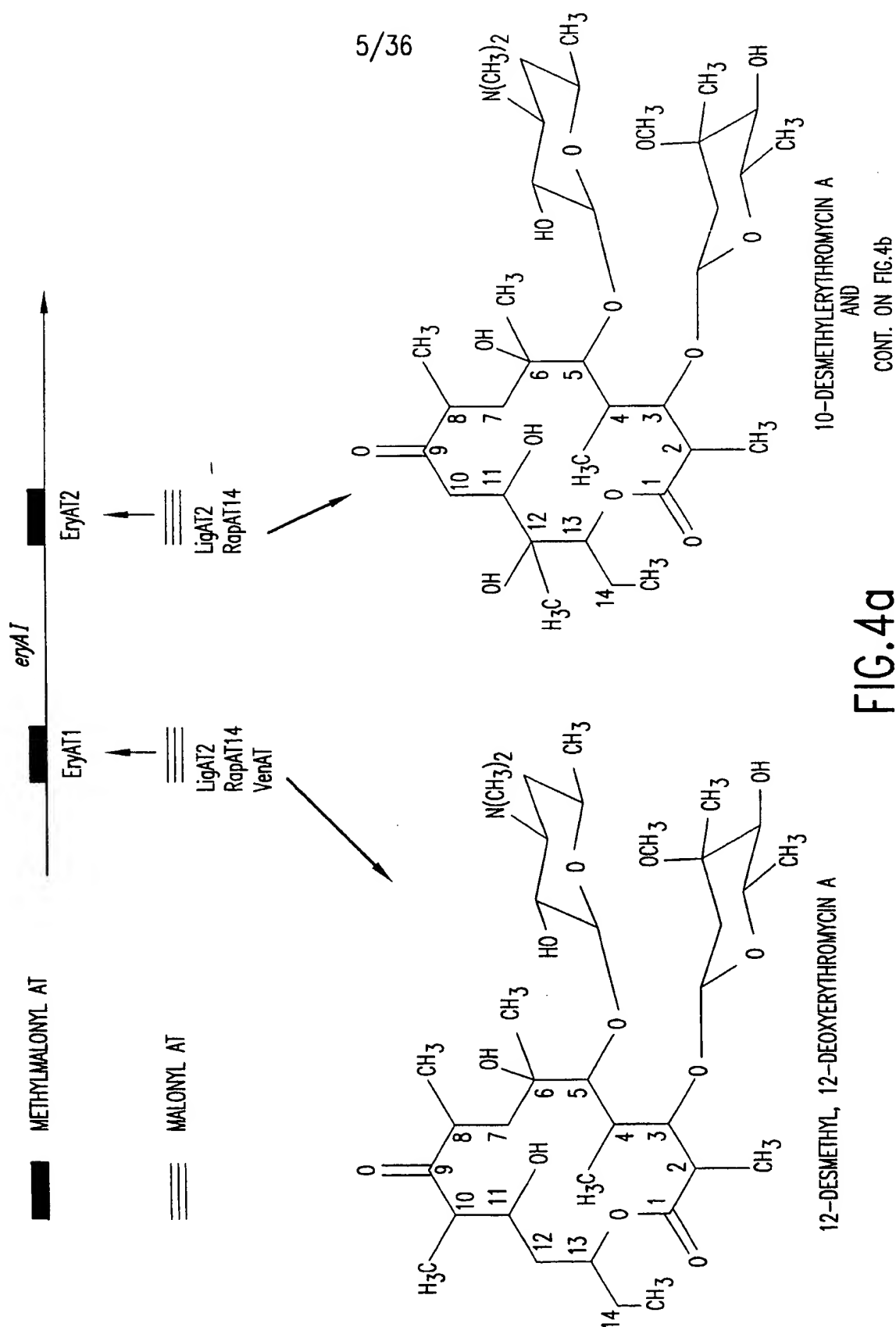
CONT. FROM FIG.4a

10-DESMETHYL-12-DEOXYERYTHROMYCIN A
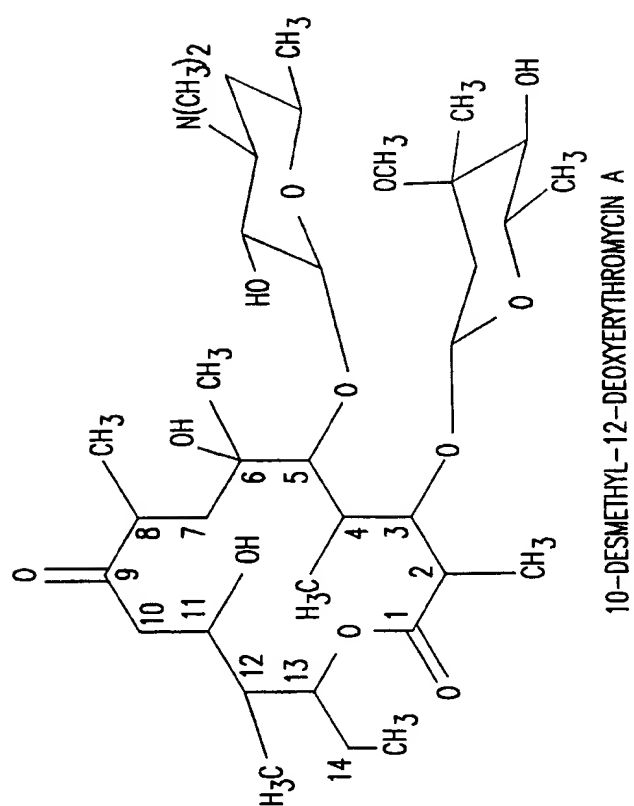
FIG.4b

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FIG.4c

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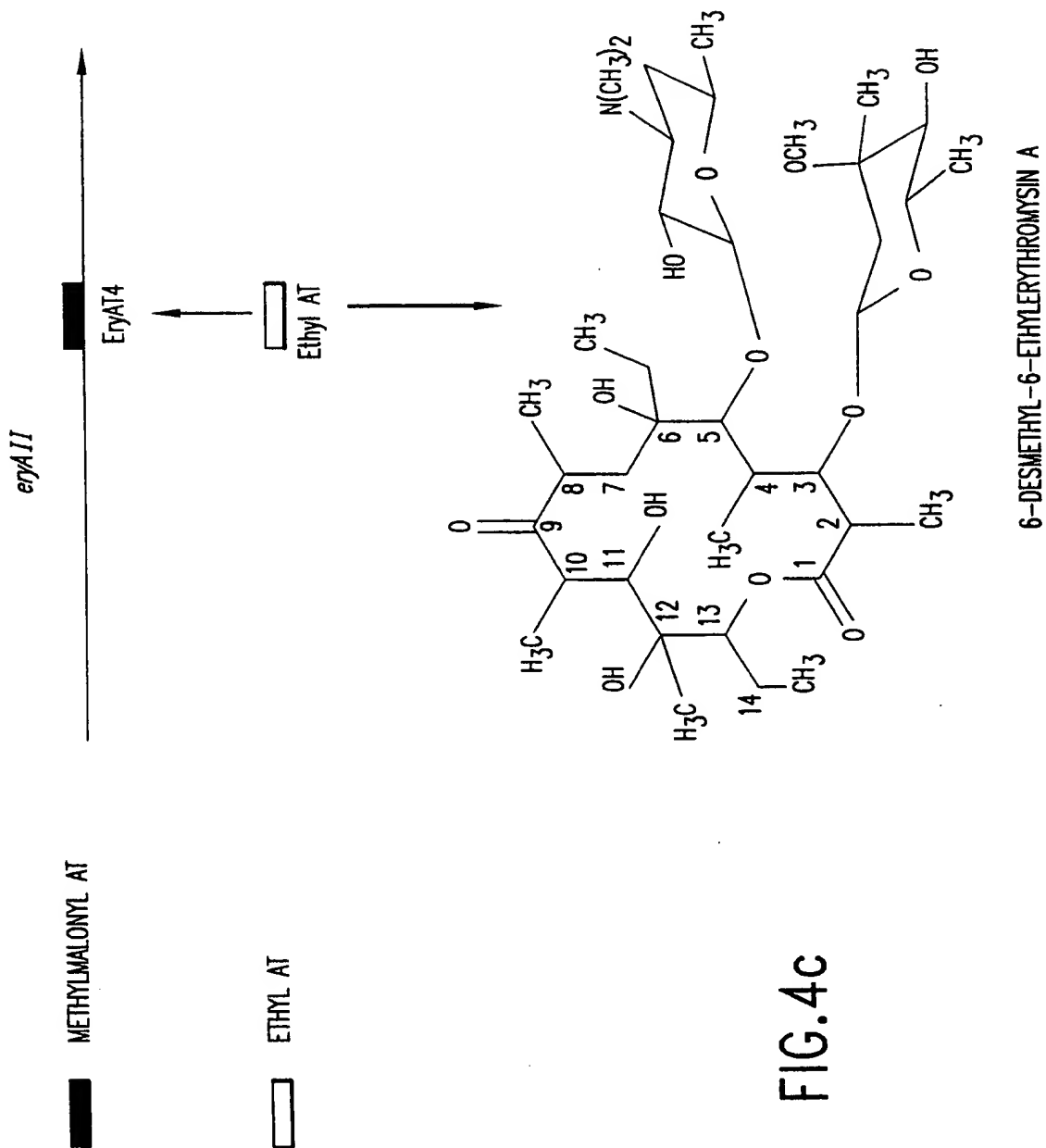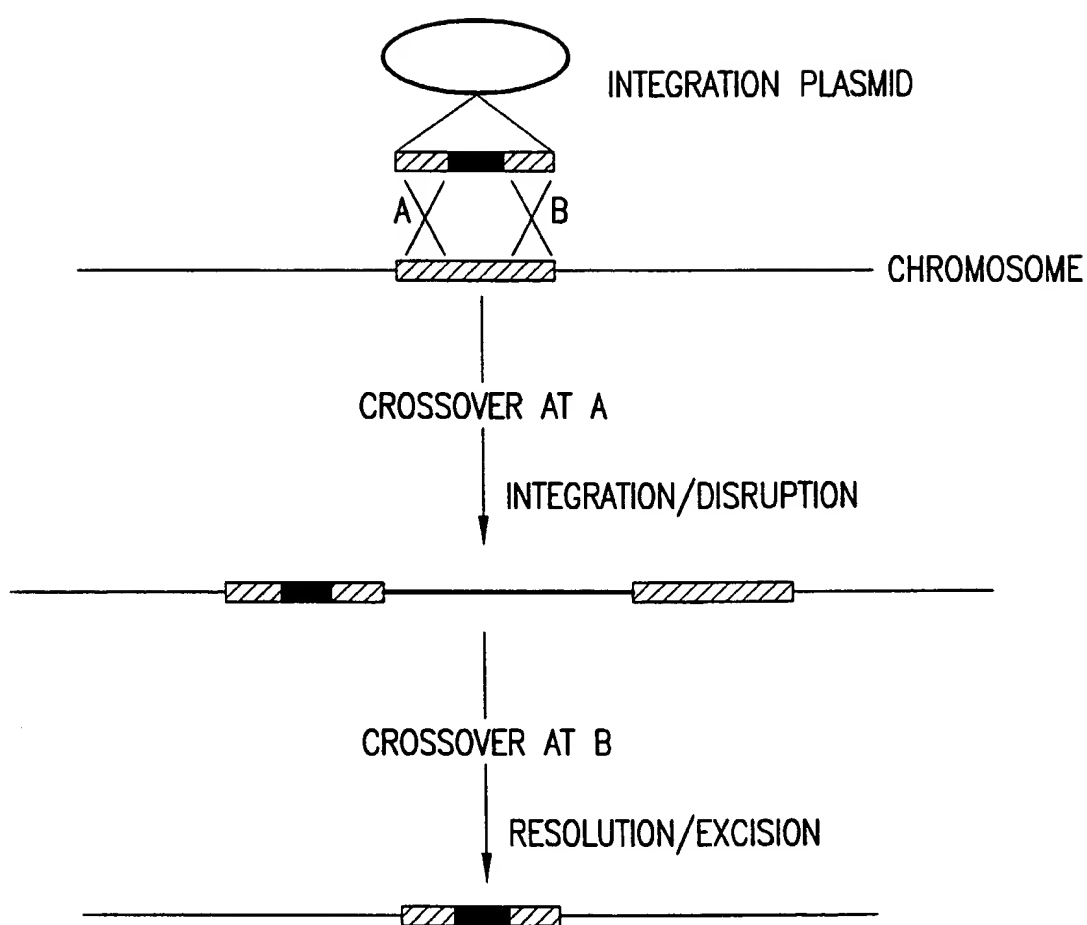
FIG.5
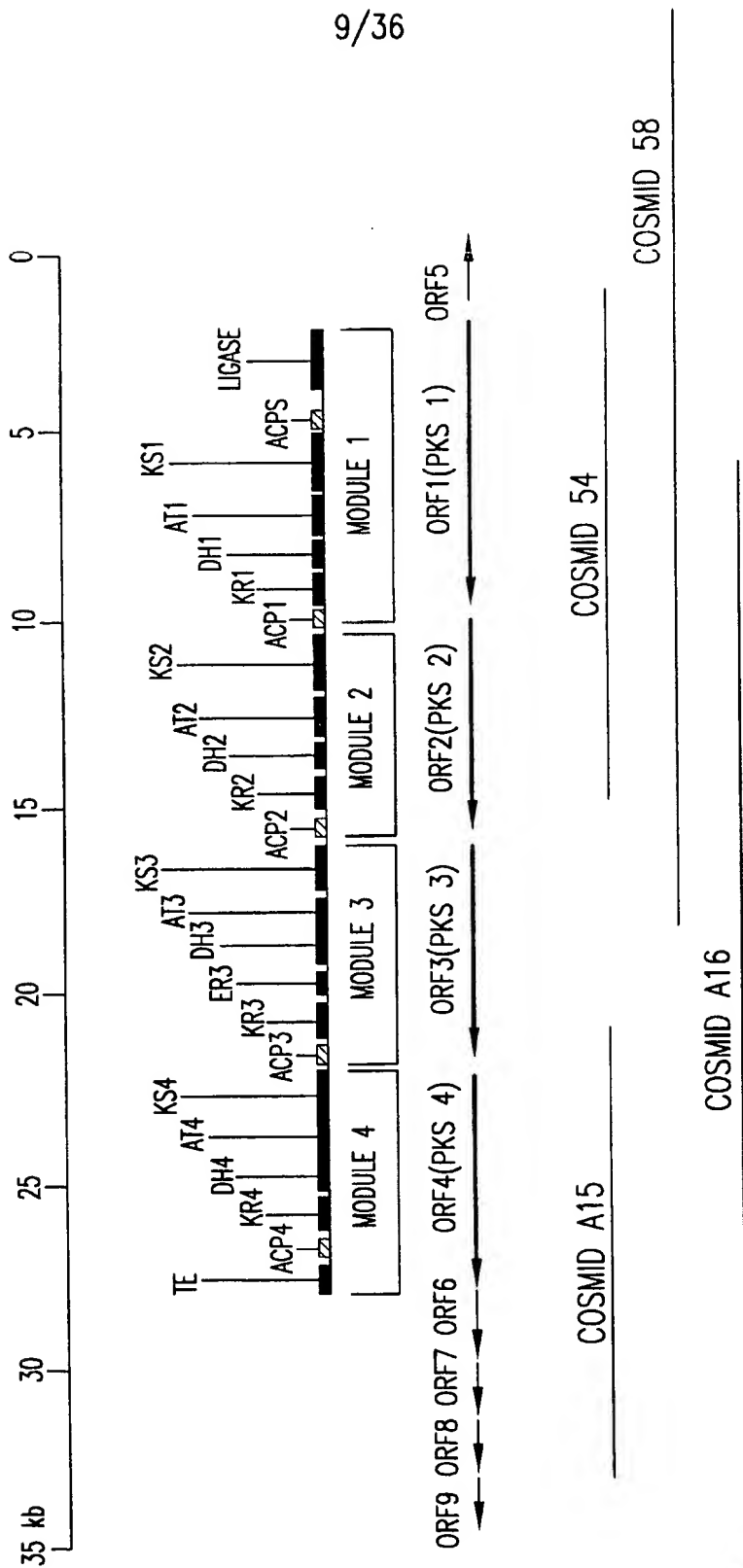
FIG.6

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GGGCCGCTGGCGGTGATGTTCACCGGACAGGGCTCCCAACGCCCCGGCATGGGACGACAG   60
G   P   L   A   V   M   F   T   G   Q   G   S   Q   R   P   G   M   G   R   Q    20

TTGTACGAGCACTTCCCCGTCTSCGCCCAGGCACTGGACGAGGTCTTCGCACTCGCCACC  120
L   Y   E   H   F   P   V   F   A   Q   A   L   D   E   V   F   A   L   A   T    40

CCCGGACTACGCGAGGTGATGTTCGACCCCGACCAGGCCGAAACACTCCAACGCACCGAC  180
P   G   L   R   E   V   M   F   D   P   D   Q   A   E   T   L   Q   R   T   D    60

CACGCCCAGATCGCCCTGTTCGCCTTCGAAACCGCCCTCTACCGACTCTGGGAATCCTGG  240
H   A   Q   I   A   L   F   A   F   E   T   A   L   Y   R   L   W   E   S   W    80

GGCCTGCGACCCGACATGGTCTGCGGACACTCGGTCGGAGAAATCACCGCAGCCCACGTC  300
G   L   R   P   D   M   V   C   G   H   S   V   G   E   I   T   A   A   H   V    100

TCCGGCACCCTCACCCTCCCCGACGCCGTCCACCTCGTCACCACACGCGGCACCCTCATG  360
S   G   T   L   T   L   P   D   A   V   H   L   V   T   T   R   G   T   L   M    120

CAAAACCTGCCCCCCGGCGGCGCCATGCTCGCCGTCGCCACCGACCCCCACACCCTCCAA  420
Q   N   L   P   P   G   G   A   M   L   A   V   A   T   D   P   H   T   L   Q    140

CCCCACCTCGACAACCACCACGACACCATCTCCATCGCCGCCATCAACGGCCCCCACGCC  480
P   H   L   D   N   H   H   D   T   I   S   I   A   A   I   N   G   P   H   A    160

ACCGTCCTCTCCGGCGACCGCACCACCCTCCACCACATCGCCACCCAACTCAACACCAAA  540
T   V   L   S   G   D   R   T   T   L   H   H   I   A   T   Q   L   N   T   K    180

ACCAACTGGCTCAACGTCAGCCACGCCTTCCACTCCCCCCCTCATGCAACCCATCCTCCAA  600
T   N   W   L   N   V   S   H   A   F   H   S   P   L   M   Q   P   I   L   Q    200

CCCTTCACCACCACCCTCAACACCCTCACCCACCACCCCCCACACACACCCCTCATCAGC  660
P   F   T   T   T   L   N   T   L   T   H   H   P   P   H   T   P   L   I   S    220

ATGCTCACCGCCACACCCACCCACCCCGACACCACCCACTGGACCCAGCACATCACCGCA  720
M   L   T   A   T   P   T   H   P   D   T   T   H   W   T   Q   H   I   T   A    240

CCCGTCCGCTACACCGACACCCTCCACCACCTCCACCACCACGGCATCACCACCTACCTC  780
P   V   R   Y   T   D   T   L   H   H   L   H   H   H   G   I   T   T   Y   L    260

GAAATCGGCCCCGACACCACCCTCACCGCCCTCGCCCGCACCACCCTCCCCACCACCACC  840
E   I   G   P   D   T   T   L   T   A   L   A   R   T   T   L   P   T   T   T    280

CACCTCATCCCCACCACCCGCCGCAACCACAACGAAGTCCGCAGCACGAACGAGGCGTTG  900
H   L   I   P   T   T   R   R   N   H   N   E   V   R   S   T   N   E   A   L    300

GGCAGGGTGTTCAGCGTGGGCCACTCGGTGGACTGGCGGGCCCTCACTCCGACCGGGAGG  960
G   R   V   F   S   V   G   H   S   V   D   W   R   A   L   T   P   T   G   R    320

CGTACCTCCCTGCCGACGTACCCCT                                     985
R   T   S   L   P   T   Y   P                                  328
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FIG.7

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PCR OLIGOS:

AvrII

N-TERMINAL OLIGO: 5' EcoRI Tag–CCTAG GCTGGCGGTGATGTTCA–3'
                                GGGCC

| ENGINEERED AvrII | | HOMOLOGOUS REGION |

Nsi I

C-TERMINAL OLIGO: 5' BamHI Tag–ATGCAT ACGTCGGCAGGGAGGTAC–3'
                              G GG

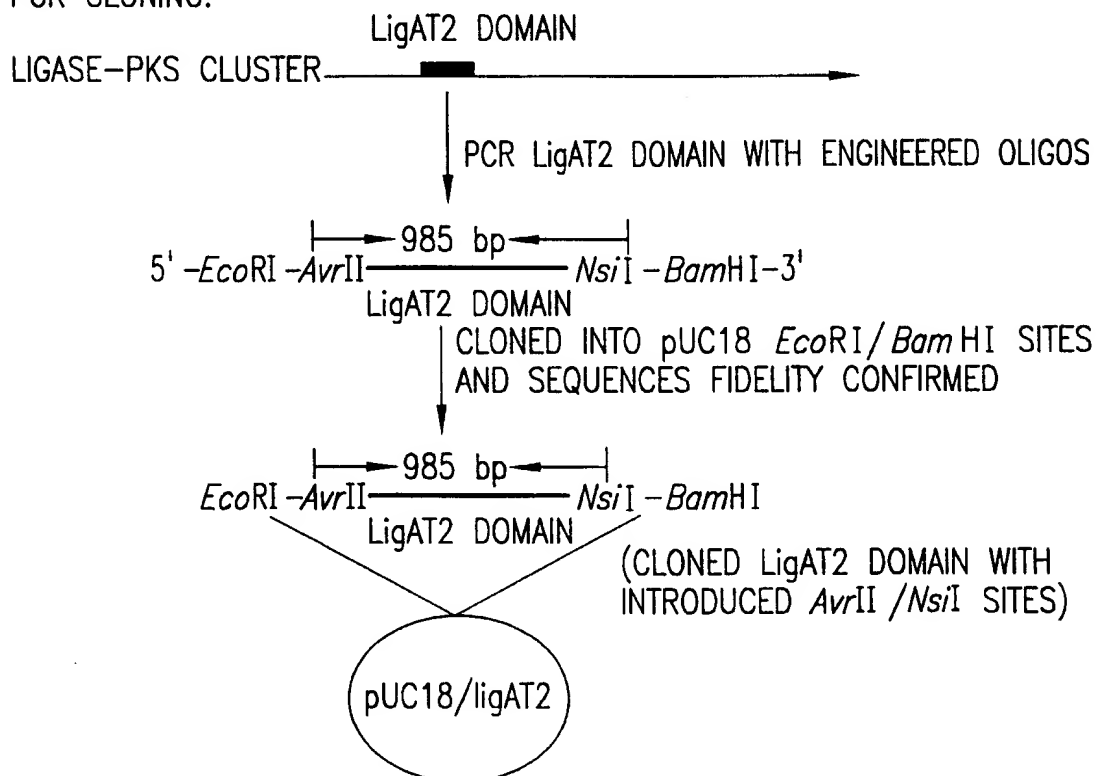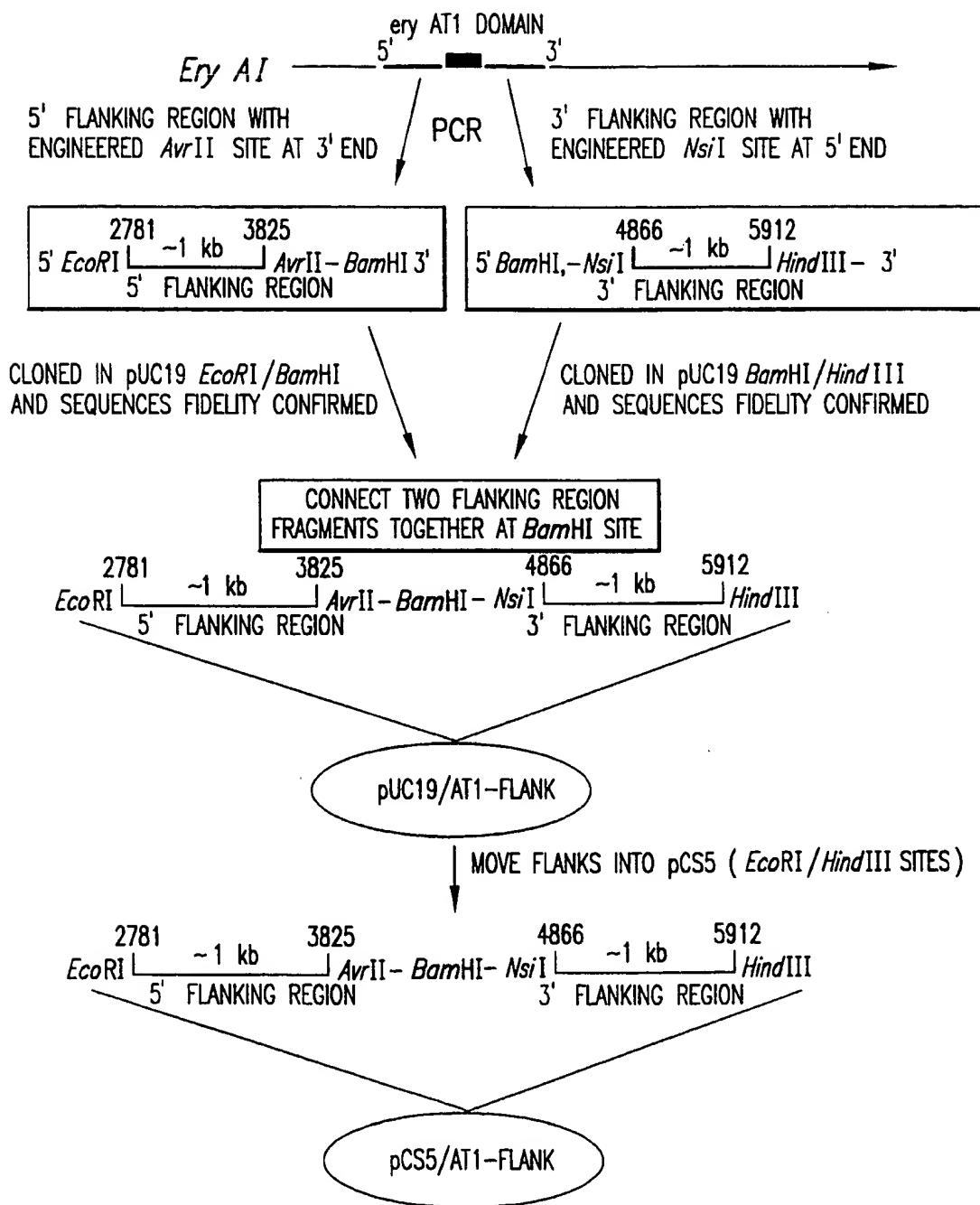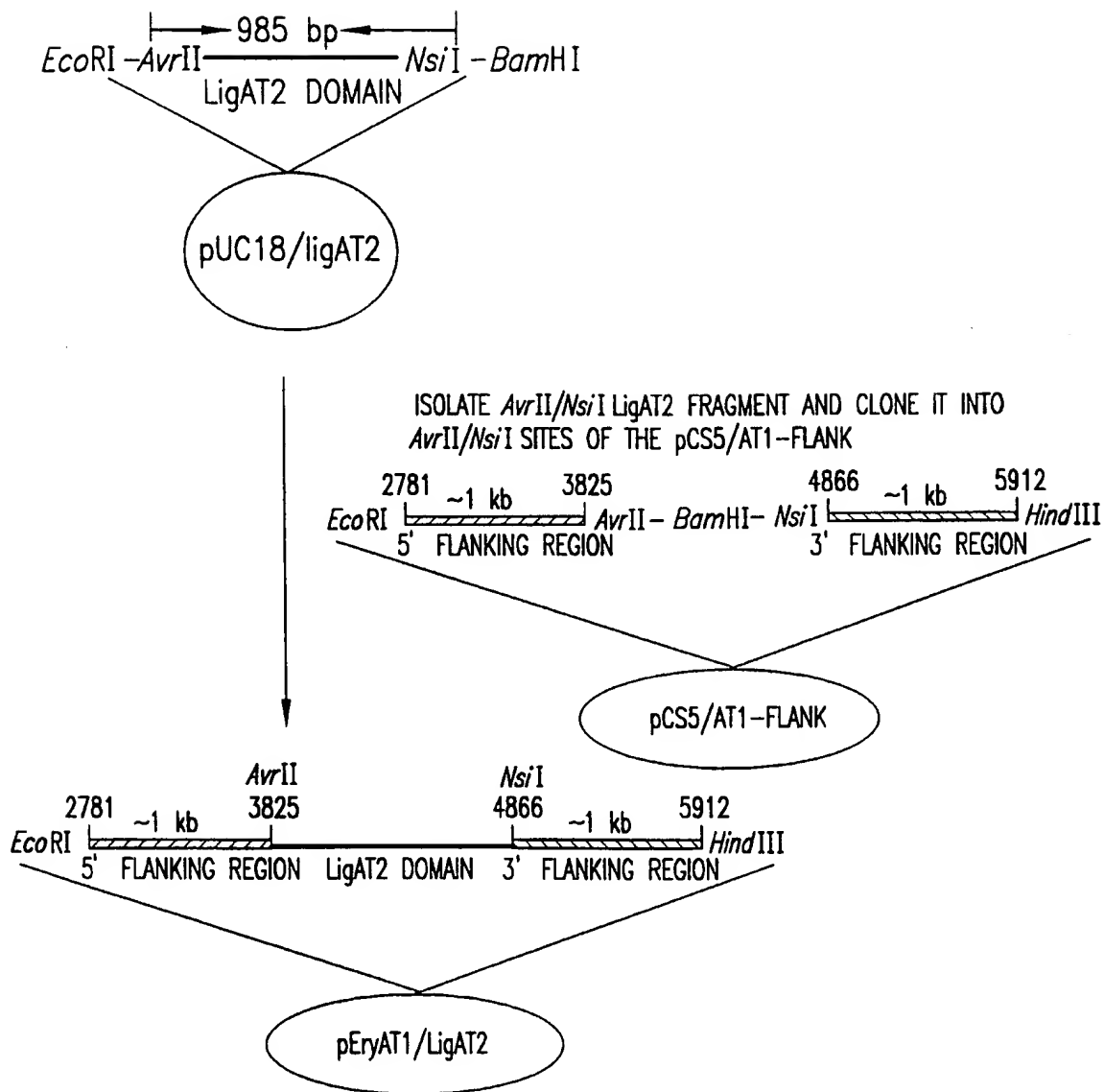| ENGINEERED Nsi I | | HOMOLOGOUS REGION |

PCR CLONING:

                                LigAT2 DOMAIN
LIGASE–PKS CLUSTER ─────────────■──────────────────────────►

              │ PCR LigAT2 DOMAIN WITH ENGINEERED OLIGOS
              ▼

              ├──► 985 bp ◄──┤
5' –EcoRI –AvrII────────────── Nsi I –BamHI–3'
              LigAT2 DOMAIN

              │ CLONED INTO pUC18 EcoRI/BamHI SITES
              │ AND SEQUENCES FIDELITY CONFIRMED
              ▼

              ├──► 985 bp ◄──┤
EcoRI –AvrII────────────── Nsi I –BamHI
              LigAT2 DOMAIN
                                    (CLONED LigAT2 DOMAIN WITH
                                    INTRODUCED AvrII /Nsi I SITES)

                          ⬭ pUC18/ligAT2 ⬭

FIG.8

FIG.9

FIG.10

CONSTRUCTION OF ery AT2 FLANKING REGIONS IN pCS5
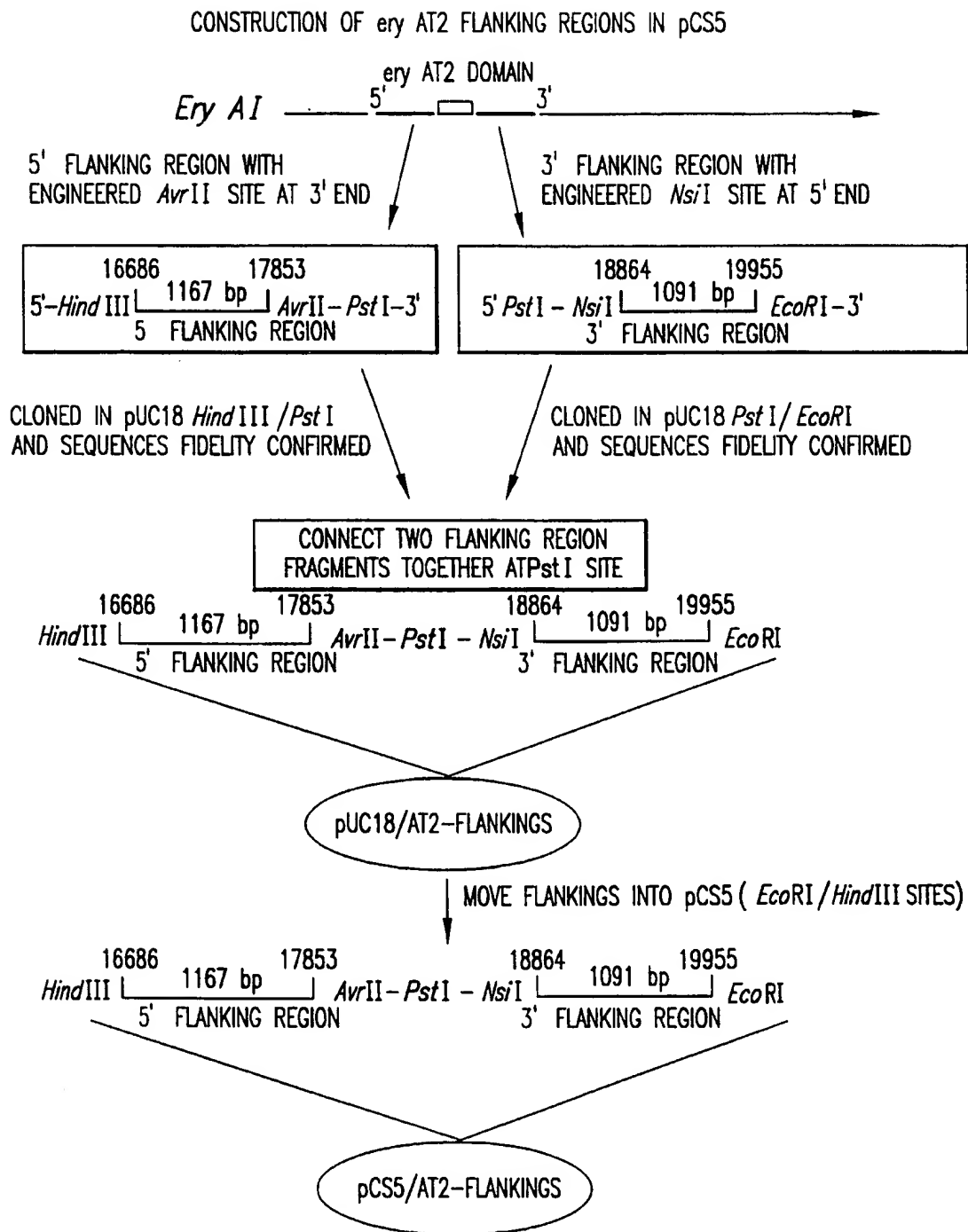
FIG.11

SCHEME FOR CONSTRUCTION OF pEryAT2/LigAT2 INTEGRATION PLASMID
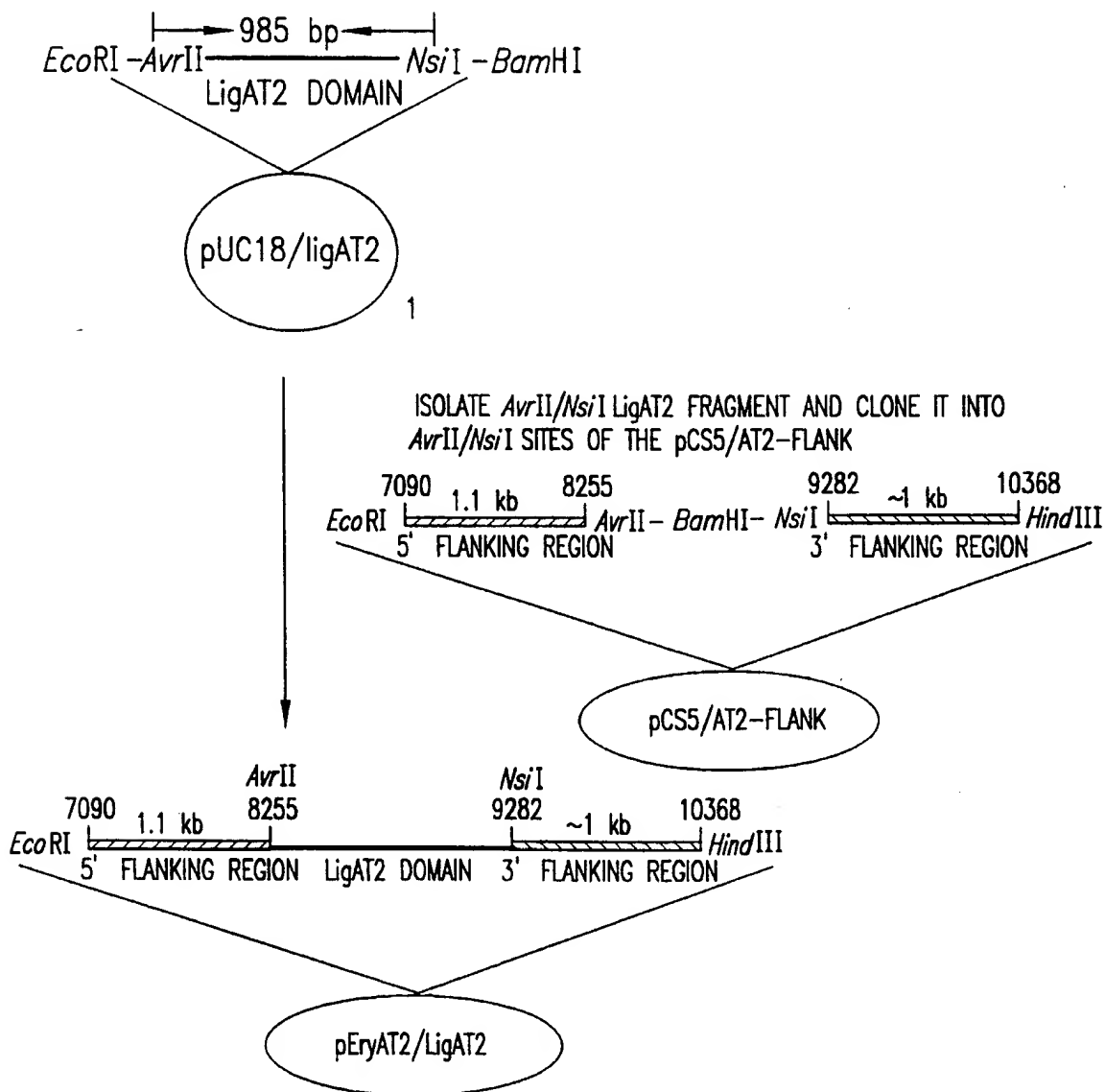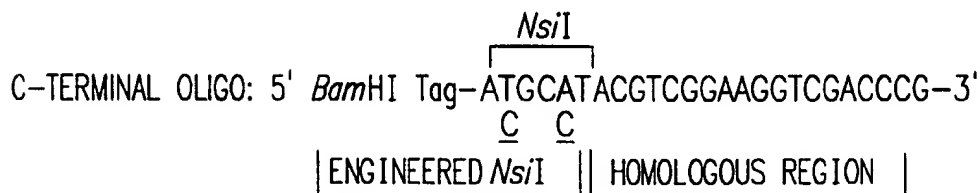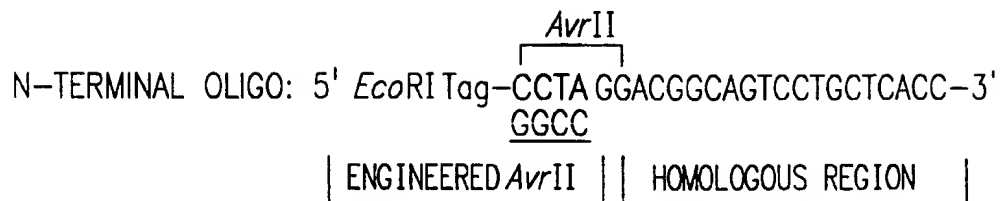

FIG.12

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```
CCTAGGACGGCAGTCCTGCTCACCGGGCAGGGTTCCCAGCGTCAGGGCATGGGGCGCGAA   60
 P  R  T  A  V  L  L  T  G  Q  G  S  Q  R  Q  G  M  G  R  E   20

CTGTACGACCGGTCACCGGTGTTCGCCGCCTCGTTCGACGCGATCTGCGCTCAACTCGAC  120
 L  Y  D  R  S  P  V  F  A  A  S  F  D  A  I  C  A  Q  L  D   40

GGGCAACTGCCTCGTCCCCTCAAGGACGTTCTCTTCGCCCCCGAGGGGTCGGAGGACGCC  180
 G  Q  L  P  R  P  L  K  D  V  L  F  A  P  E  G  S  E  D  A   60

GCGCTCASCGACCGTACGGTGTTCACACAGGCGGCTCTGTTCGCCGTGGAGACCTCCCTG  240
 A  L  I  D  R  T  V  F  T  Q  A  A  L  F  A  V  E  T  S  L   80

TTCCGGCTGTTCGAGGCCCACGGCCTCGSCCCCGACTACCTCASCGGCCACTCCATCGGC  300
 F  R  L  F  E  A  H  G  L  V  P  D  Y  L  I  G  H  S  I  G  100

GAAGTGACCGCGGCCCGCCTGGCCGGGGTCCTCGATCTGGCGGACGCGTGCGTCCTGGTC  360
 E  V  T  A  A  H  L  A  G  V  L  D  L  A  D  A  C  V  L  V  120

GCCCACCGCGGCCGCCTGATGCAGTCGGCCCGGGCCGGCGGCGCGATGGCCGCGGTCCAG  420
 A  H  R  G  R  L  M  Q  S  A  R  A  G  G  A  M  A  A  V  Q  140

GCGAGCGAGGACGAGGTACGCGAGGCCCTCGCGACCTTCGACGATGCGGTTCCCGTGGCC  480
 A  S  E  D  E  V  R  E  A  L  A  T  F  D  D  A  V  A  V  A  160

GGAGTCAACGGCCCGAACGCCACCGTCGTCTCCGGCGACGAGGACGCGGTCGAGCGGCTG  540
 G  V  N  G  P  N  A  T  V  V  S  G  D  E  D  A  V  E  R  L  180

GTCGCGCGCTGGCGCGAGCAGGGCAGGCGGACGAAGCGGCTGCCGGTCAGCCACGCCTTC  600
 V  A  R  W  R  E  Q  G  R  R  T  K  R  L  P  V  S  H  A  F  200

CACTCGCCGCACATGGACGGGATCGTCGACGAGTTCGTCACCGCCGTCTCCGGGCTCACC  660
 H  S  P  H  M  I  G  I  V  D  E  F  V  T  A  V  S  G  L  T  220

TTCCGCTCCCCGACGLTCCCGGTCGTCTCCAACGTCACCGGGACCCTCGCCACCGTCGAC  720
 F  R  S  P  T  I  P  V  V  S  N  V  T  G  T  L  A  T  V  D  240

CACCTGACCTCGCCCGCGTACTGGGCACGCCACATCCGCGAGGCCGTGCGCTTCGCCGAC  780
 Q  L  T  S  P  A  Y  W  A  R  H  I  R  E  A  V  R  F  A  D  260

GGGGTGCGGTACCTGGAGGGCGAGGGCGTCACCGAATGGCTGGAGCTCGGGCCCGACGGC  840
 G  V  R  Y  L  E  G  E  G  V  T  E  W  L  E  L  G  P  D  G  230

GTTCTCGTCGCCCTGGTCGAGGACTGCCTGGCGAAGGAGGCGGGATCGCTCGCGTCCGCC  900
 V  L  V  A  L  V  E  D  C  L  A  K  E  A  G  S  L  A  S  A  300

CTGCGCAAGGGGGCGAGCGAGCCCCACACCGTGGGCGCGGCCATGGCCCGCGCGGTGCTG  960
 L  R  K  G  A  S  E  P  H  T  V  G  A  A  M  A  R  A  V  L  320

CGCGGATCCGGCCCCGACTGGGCGGCGGTGTTCCCCGGCGCACGGCGGGTCGACCTTCCG 1020
 R  G  S  G  P  D  W  A  A  V  F  P  G  A  R  R  V  D  L  P  340

ACGTATGCAT                                                    1030
 T  Y  A                                                       343
```
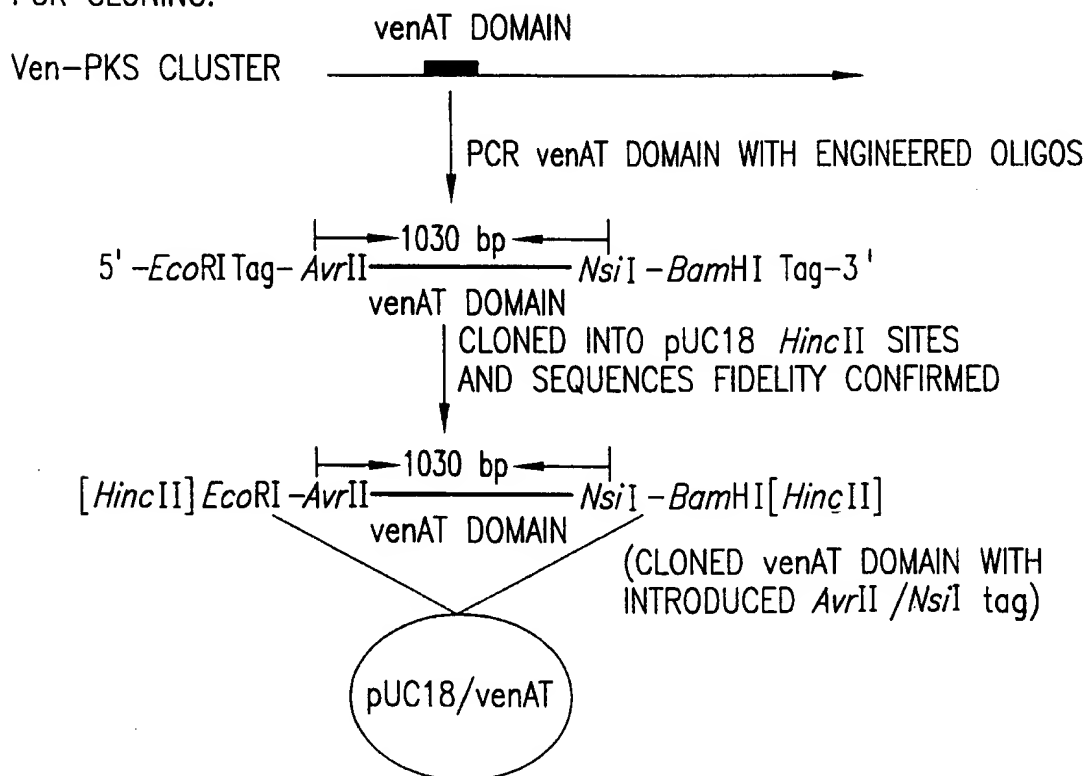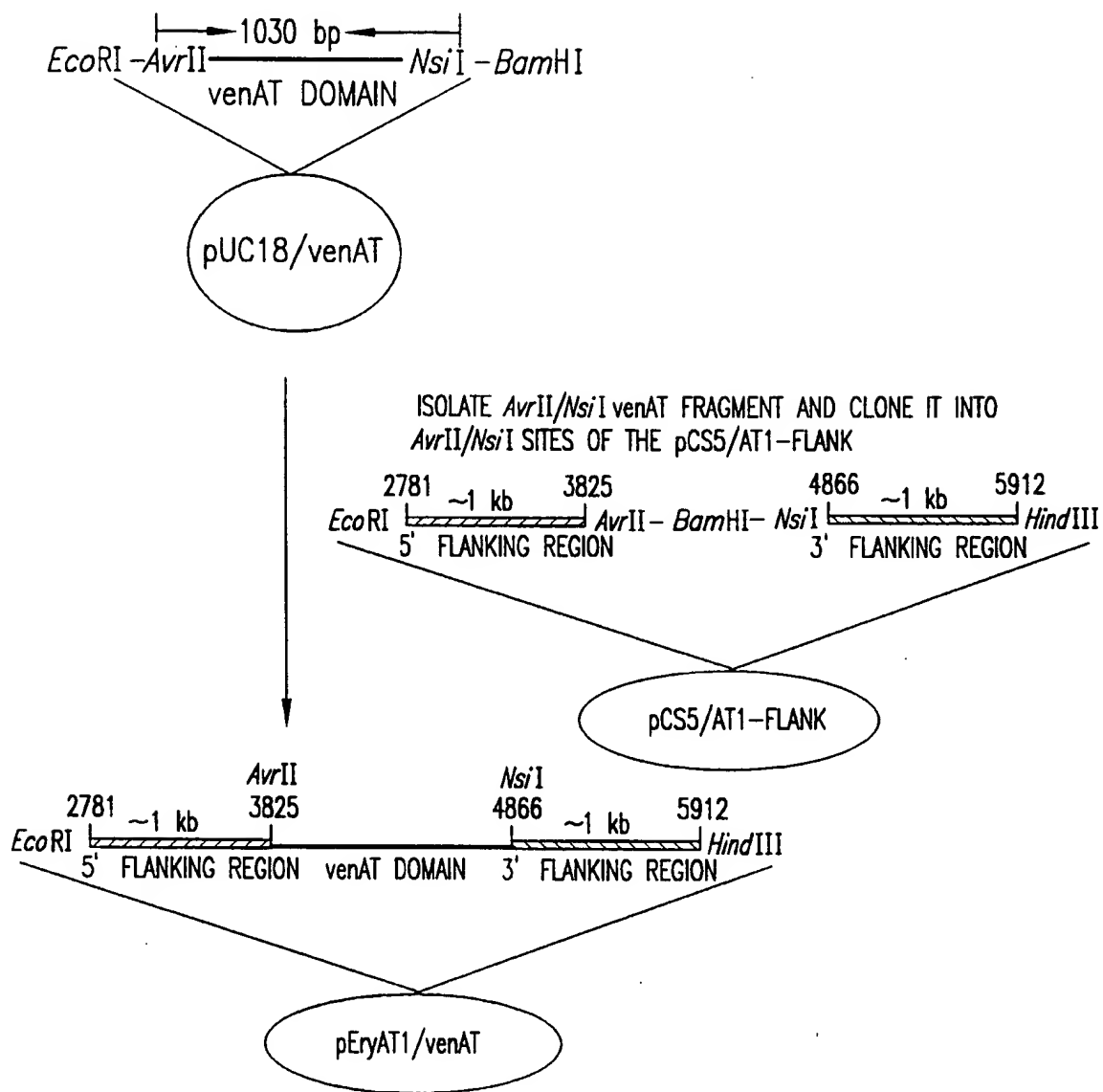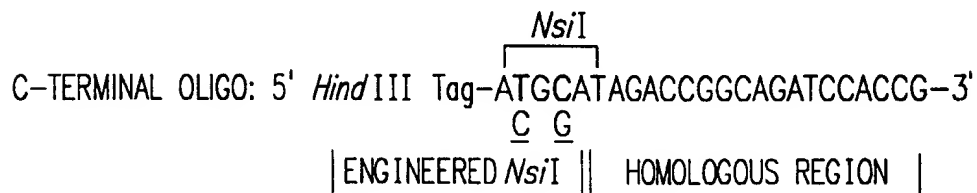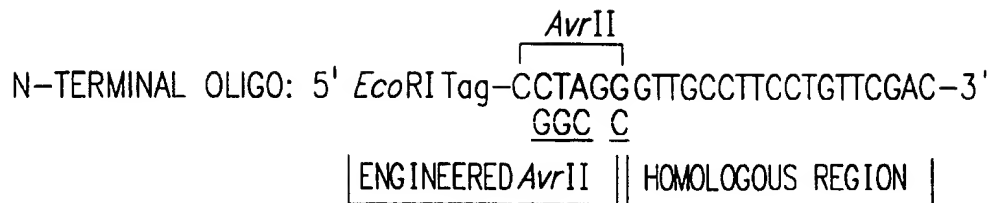
FIG.13

PCR OLIGOS:

AvrII
N-TERMINAL OLIGO: 5' _Eco_RI Tag—CCTA GGACGGCAGTCCTGCTCACC—3'
                              GGCC

| ENGINEERED _Avr_II || HOMOLOGOUS REGION |

Nsi I
C-TERMINAL OLIGO: 5' _Bam_HI Tag—ATGCAT ACGTCGGAAGGTCGACCCG—3'
                                  C   C

| ENGINEERED _Nsi_I || HOMOLOGOUS REGION |

PCR CLONING:

                              venAT DOMAIN
Ven—PKS CLUSTER

            PCR venAT DOMAIN WITH ENGINEERED OLIGOS

                    ├——1030 bp——┤
5'—_Eco_RI Tag— _Avr_II————————— _Nsi_I —_Bam_HI Tag—3'
                    venAT DOMAIN

            CLONED INTO pUC18 _Hinc_II SITES
            AND SEQUENCES FIDELITY CONFIRMED

                    ├——1030 bp——┤
[_Hinc_II] _Eco_RI —_Avr_II————————— _Nsi_I —_Bam_HI [_Hinc_II]
                    venAT DOMAIN
                                    (CLONED venAT DOMAIN WITH
                                    INTRODUCED _Avr_II /_Nsi_I tag)

                    pUC18/venAT

FIG.14

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FIG.15

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PCR OLIGOS:

$\overset{\displaystyle Avr\text{II}}{\overbrace{}}$

N-TERMINAL OLIGO: 5' *Eco*RI Tag—CCTAGG GTTGCCTTCCTGTTCGAC—3'
                              GGC C

| ENGINEERED *Avr*II | | HOMOLOGOUS REGION |

$\overset{\displaystyle Nsi\text{I}}{\overbrace{}}$

C-TERMINAL OLIGO: 5' *Hind*III Tag—ATGCAT AGACCGGCAGATCCACCG—3'
                             C G

| ENGINEERED *Nsi*I | | HOMOLOGOUS REGION |

PCR CLONING:

                                 RapAT14 DOMAIN
RAPAMYCIN CLUSTER ——————————▬▬▬——————————▶

                    │ PCR RapAT14 DOMAIN WITH ENGINEERED OLIGOS
                    ▼

         ├——▶ 1023 bp ◀——┤
5'—*Eco*RI —*Avr*II———————————— *Nsi*I —*Hind* III—3'
         RapT14 DOMAIN
                    │ CLONED INTO pUC19 *Hinc*II SITE
                    │ ANDS SEQUENCES FIDELITY CONFIRMED
                    ▼

         ├——▶ 1023 bp ◀——┤
    *Eco*RI —*Avr*II———————————— *Nsi*I —*Hind* III
         RapAT14 DOMAIN

  Hind III ———————   ———————*Eco*RI

                   ⟨ pUC19/rapAT14 ⟩

FIG.16

FIG.17

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FIG.18

FIG.19

FIG.20

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```
GCCGACCGTGTCGTGTTCGTGTTCCCCGGCCAGGGCTCGCAGTGGGCCGGAATGGCCGAG   60
 A  D  R  V  V  F  V  F  P  G  Q  G  S  Q  W  A  G  M  A  E   20

GGGCTGCTGGAGCGGTCCGGCGCGTTCCGGAGTGCGGCCGACTCGTGCGACGCCGCGCTG  120
 G  L  L  E  R  S  G  A  F  R  S  A  A  D  S  C  D  A  A  L   40

CGGCCGTACCTCGGCTGCTCGGTGCTGAGCGTGCTGCGCGGGGGAACCGGACGCGCCCTCG  180
 R  P  Y  L  G  W  S  V  L  S  V  L  R  G  E  P  D  A  P  S   60

CTCGACCGGGTCGACGTCGTGCAGCCGGTGCTGTTCACGATGATGGTCTCGCTCGCGGCG  240
 L  D  R  V  D  V  V  Q  P  V  L  F  T  M  M  V  S  L  A  A   80

GTCTGGCGTGCGCTGGGCGTGGAACCGGCGGCGGTCGTCGGGCACTCGCAGGGTGAGATC  300
 V  W  R  A  L  G  V  E  P  A  A  V  V  G  H  S  Q  G  E  I  100

GCCGCTGCCCATGTCGCCGGTGCGCTGTCGCTGGACGACTCGGCCCGGATCGTCGCCCTG  360
 A  A  A  H  V  A  G  A  L  S  L  D  D  S  A  R  I  V  A  L  120

CGCAGTCGGGCGTGGCTCGGACTGGCGGGCAAGGGCGGCATGGTGGCGGTGCCGATGCCG  420
 R  S  R  A  W  L  G  L  A  G  K  G  G  M  V  A  V  P  M  P  140

GCGGAGGAGCTGCGGCCGCGGCTGGTGACGTGGGGGGGACCGTCTGGCCGTCGCCGCCGTC  480
 A  E  E  L  R  P  R  L  V  T  W  G  D  R  L  A  V  A  A  V  160

AACAGCCCCGGTTCCTGCGCCGTCGCAGGCGACCCGGAGGCGCTGGCCGAACTGGTGGCG  540
 N  S  P  G  S  C  A  V  A  G  D  P  E  A  L  A  E  L  V  A  180

CTGCTGACCGGTGAGGGGGTGCACGCCCGGCCGATCCCCGGCGTCGACACGGCGGGCCAC  600
 L  L  T  G  E  G  V  H  A  R  P  I  P  G  V  D  T  A  G  H  200

TCGCCGCAGGTGGACGCGTTGCGGGCTCATCTGCTGGAGGTGCTGGCCCCCGGTCGCCCCC  660
 S  P  Q  V  D  A  L  R  A  H  L  L  E  V  L  A  P  V  A  P  220

CGACCGGCCGACATCCCGTTCTACTCGACGGTGACCGGCGGGCTGCTGGACGGCACCGAG  720
 R  P  A  D  T  P  F  Y  S  T  V  T  G  G  L  L  D  G  T  E  240

CTGGACGCGACGTACTGGTACCGCAACATGCGCGAGCCCGTCGAGTTCGAGCGGGCCACA  780
 L  D  A  T  Y  W  Y  R  N  M  R  E  P  V  E  F  E  R  A  T  260

CGGGCGCTGATCGCCGACGGGCACGACGTCTTCCTGGAGACGAGCCCGCATCCCATGCTG  840
 R  A  L  I  A  D  G  H  D  V  F  L  E  T  S  P  H  P  M  L  280

GCCGTGGCGCTGGAGCAGACGGTCACCGACGCCGGCACCGACGCGGCGGTGCTCGGGACC  900
 A  V  A  L  E  Q  T  V  T  D  A  G  T  D  A  A  V  L  G  T  300

CTGCGCCGCCGCCACGGCGGTCCTCGCGCGCTGGCCCTGGCCGTCTGCCGCGCCTTCGCG  960
 L  R  R  R  H  G  G  P  R  A  L  A  L  A  V  C  R  A  F  A  320

CACGGCGTGGAGGTGGACCCCGAGGCGGTCTTCGGTCCGGGCGCACGGCCCGTGGAGTTG 1020
 H  G  V  E  V  D  P  E  A  V  F  G  P  G  A  R  P  V  E  L  340

CCCACCTATCCG                                                  1032
 P  T  Y  P                                                    344
```
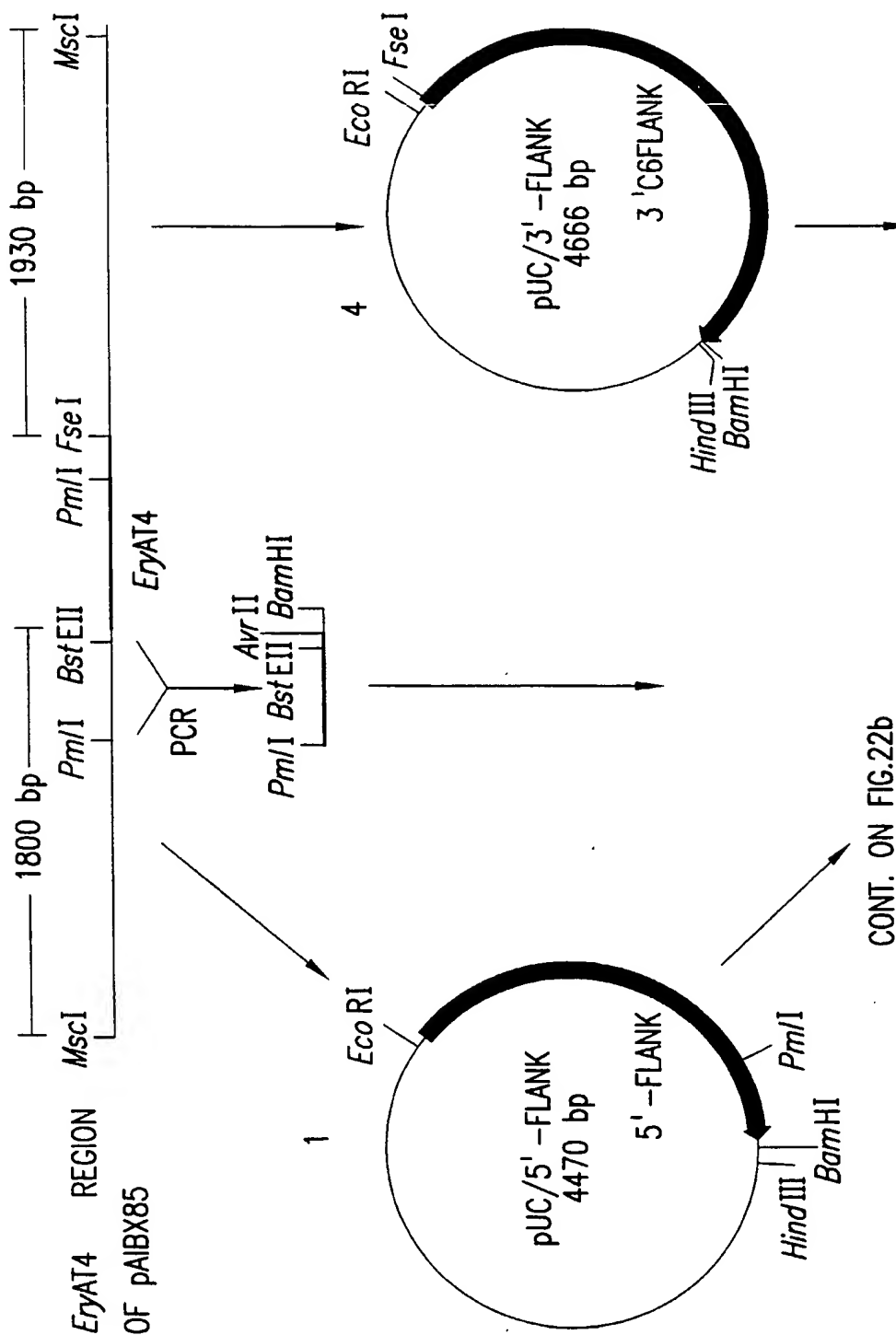
FIG.21

FIG.22a
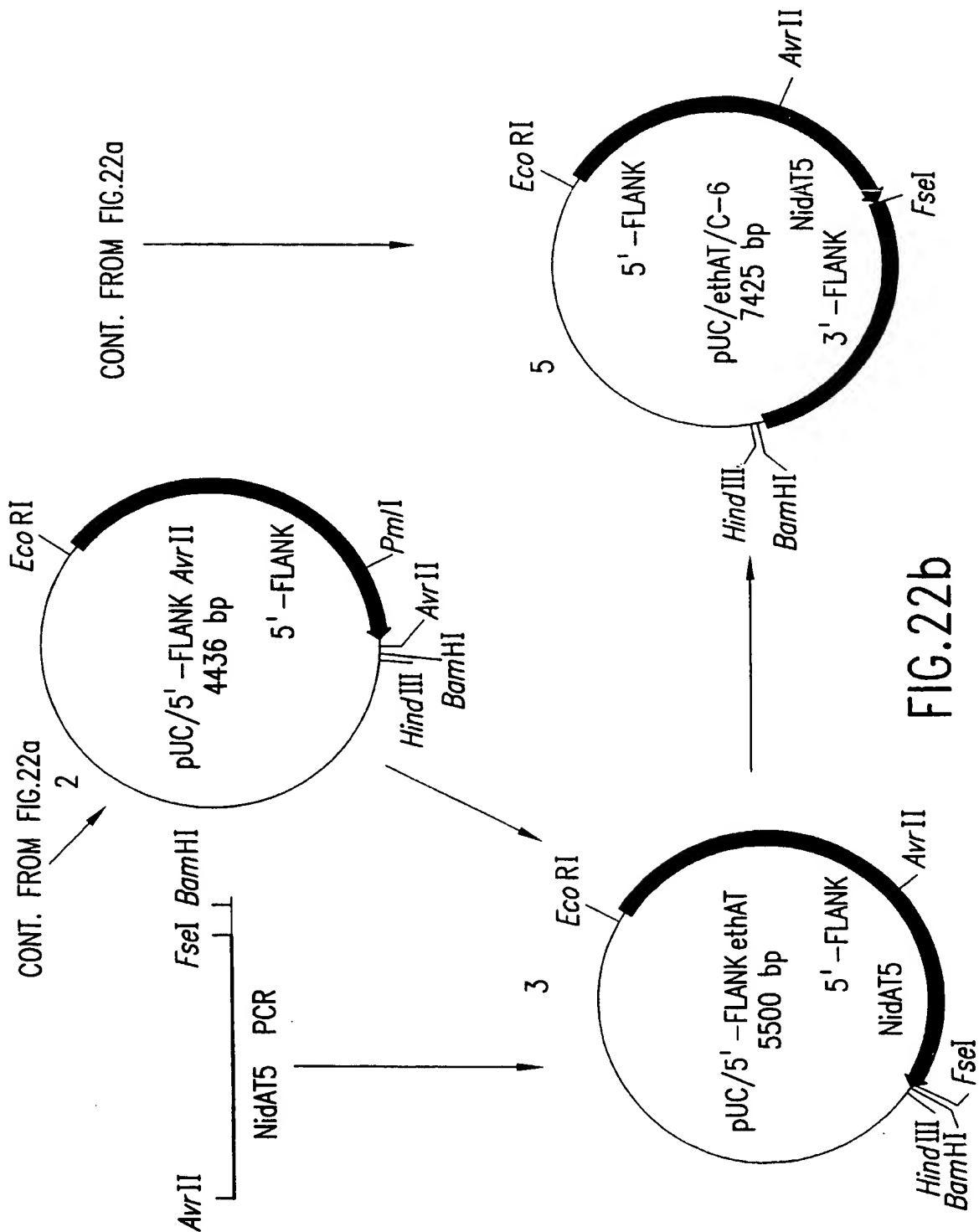
FIG.22b

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PROTEIN SEQUENCE   S   A   P   R   K   P

ORIGINAL SEQUENCE   TCCGCGCCGCGCAAGCCG

↓ ↓ ↓

ALTERED SEQUENCE   TCCGCGCCTAGGAAGCCG

*Avr*II SITE

PCR OLIGOS FOR 5'-FLANK *Avr*II SITE

5'- FLANK SEQUENCE

N-TERMINAL OLIGO 5'-GAGAGAGGAACCAACGCGCACGTGATCGTCGAAGAGGCACCAGC
(SEQ. ID. NO. 21)

*Pm*II SITE

5'-FLANK SEQUENCE

C-TERMINAL OLIGO 5'-GAGAGAGGATCCGACCTAGGCGCGGAGGTCACCGGCGCGACGGCG
(SEQ. ID. NO. 22)

*Bam*HI SITE   *Avr*II SITE

PCR OLIGOS FOR NidAT5 FRAGMENT

BEGINNING OF NidAT5

N-TERMINAL OLIGO 5'-GAGAGACCTAGGAAGCCGGTGTTCGTGTTCCCCGGCCAGGGCT
(SEQ. ID. NO. 23)

*Avr*II SITE

3' END OF NidAT5

C-TERMINAL OLIGO 5'-GAGAGAGGATCCGAGGCCGGCCGTGCGCCCGGACCGAAGACCGCCTC
(SEQ. ID. NO. 24)

*Bam*HI SITE   *Fse*I SITE

FIG.23
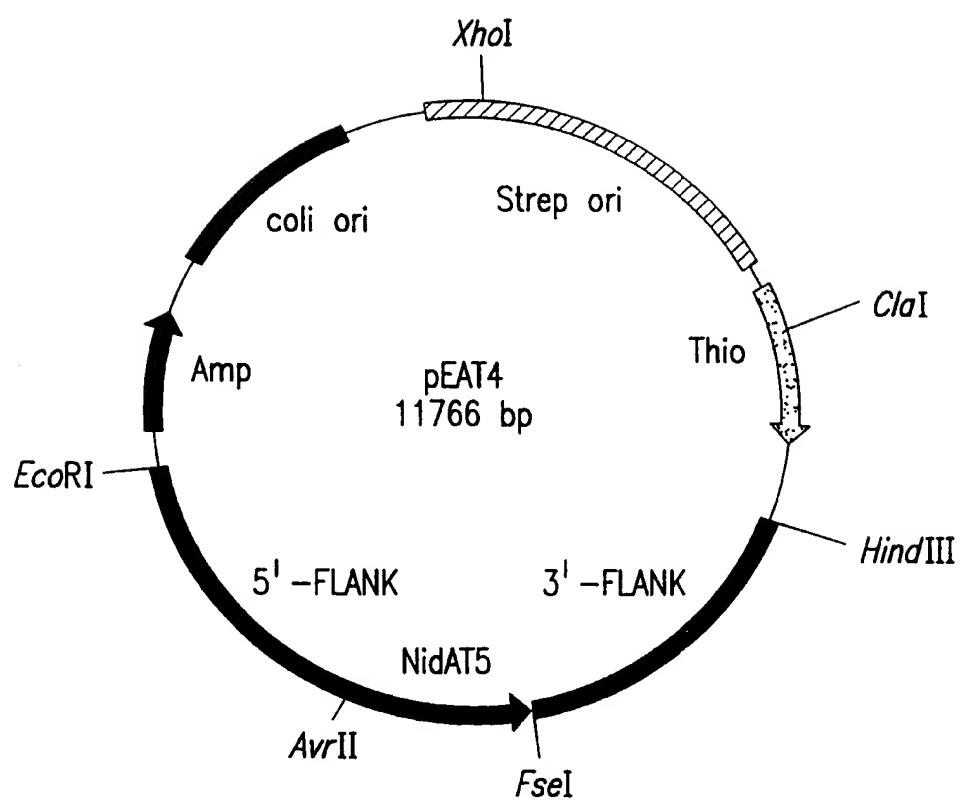
FIG.24

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```
CGCGCGCCTGCCTTCGTCTTTCCCGGGCAGGGCGCCCAGTGGGCCGGACTGGGAGCGCGG 60
R   A   P   A   F   V   F   P   G   Q   G   A   Q   W   A   G   L   G   A   R   20

CTCCTCGCGGACTCCCCCGTCTTCCGCGCCAGGGCCGAGGCATGCGCGCGGGCGCTGGAG 120
L   L   A   D   S   P   V   F   R   A   R   A   E   A   C   A   R   A   L   E   40

CCTCACCTCGACTGGTCGGTCCTCGACGTGCTGGCCGGCGCCCCGGGCACCCCTCCCATC 180
P   H   L   D   W   S   V   L   D   V   L   A   G   A   P   G   T   P   P   I   60

GACCGGGCCGACGTGGTGCAGCCGGTGCTGTTCACCACGATGGTCTCGCTGGCCGCCCTC 240
D   R   A   D   V   V   Q   P   V   L   F   T   T   M   V   S   L   A   A   L   80

TGGGAGGCCCACGGGGTGCGGCCGGCCGCGGTCGTGGGCCACTCCCAGGGCGAGGTGGCC 300
W   E   A   H   G   V   R   P   A   A   V   V   G   H   S   Q   G   E   V   A   100

GCGGCCTGCGTGGCCGGTGCCCTGTCGCTGGACGACGCTGCCCTGGTGATCGCCGGACGC 360
A   A   C   V   A   G   A   L   S   L   D   D   A   A   L   V   I   A   G   R   120

AGCAGGCTGTGGGGGCGGCTGGCCGGGAACGGCGGGATGCTCGCGGTGATGGCTCCGGCC 420
S   R   L   W   G   R   L   A   G   N   G   G   M   L   A   V   M   A   P   A   140

GAGCGGATCCGTGAGCTGCTCGAACCATGGCGGCAGCGGATTTCGGTGGCGGCGGTCAAT 480
E   R   I   R   E   L   L   E   P   W   R   Q   R   I   S   V   A   A   V   N   160

GGCCCCGCCTCGGTCACCGTCTCCGGTGACGCGCTCGCGCTGGAGGAGTTCGGCGCGCGG 540
G   P   A   S   V   T   V   S   G   D   A   L   A   L   E   E   F   G   A   R   180

CTCTCCGCCGAGGGGGTGCTGCGCTGGCCGCTGCCGGGCGTCGACTTCGCCGGCCACTCG 600
L   S   A   E   G   V   L   R   W   P   L   P   G   V   D   F   A   G   H   S   200

CCGCAGGTGGAGGAGTTCC GC5CTGAGCTCCTGGACCTGCTCTCCGGCGTACGGCCGGC 660
P   Q   V   E   E   F   R   A   E   L   L   D   L   L   S   G   V   R   P   A   220

CCTTCGCGGATACCTTTCTTCTCCACCGTGACGGCGGGTCCTTGCGGCGGCGACCAGCTG 720
P   S   R   I   P   F   P   S   T   V   T   A   G   P   C   G   G   D   Q   L   240

GACGGGGCGTACTGGTACCGCAACACGCGCGAACCCGTGGAGTTCGACGCCACGGTCCGG 780
D   G   A   Y   W   Y   R   N   T   R   E   P   V   E   F   D   A   T   V   R   260

GCGCTGCTGCGTGCGGGCCATCACACGTTCATCGAGGTCGGTCCGCATCCGCTGCTCAAC 840
A   L   L   R   A   G   H   H   T   F   I   E   V   G   P   H   P   L   L   N   280

GCCGCGATCGACGAGATCGCAGCGGACGAGGGGGTAGCGGCCACGGCCCTGCATACGCTC 900
A   A   I   D   E   I   A   A   D   E   G   V   A   A   T   A   L   H   T   L   300

CAGCGGGGCGCTGGCGGCCTTGACCGCGTGCGCAACGCGGTGGGCGCCGCTTTCGCGCAC 960
Q   R   G   A   G   G   L   D   R   V   R   N   A   V   G   A   A   F   A   H   320

GGTGTCCGGGTCGACTGGAACGCCCTGTTCGAGGGCACCGGTGCGCGCAGGGTGCCGCTT 1020
G   V   R   V   D   W   N   A   L   F   E   G   T   G   A   R   R   V   P   L   340

CCCTCGTACGCCTTC                                                    1035
P   S   Y   A   F                                                  345
```

FIG.25

PCR OLIGOS:

AvrII

N-TERMINAL OLIGO: 5' *Eco*RI Tag-CCTAGGGTCGCCTTCGTCTTTCCCGGGCAGG-3'
GCGC CCT

| ENGINEERED *Avr*II AND Val CODON | HOMOLOGOUS REGION |

NsiI

C-TERMINAL OLIGO: 5' *Bgl*II Tag-ATGCATACGAGGGAAGCGGCACCCTGC-3'
G G

| ENGINEERED *Nsi*I | HOMOLOGOUS REGION |

PCR CLONING:

NidAT6 DOMAIN

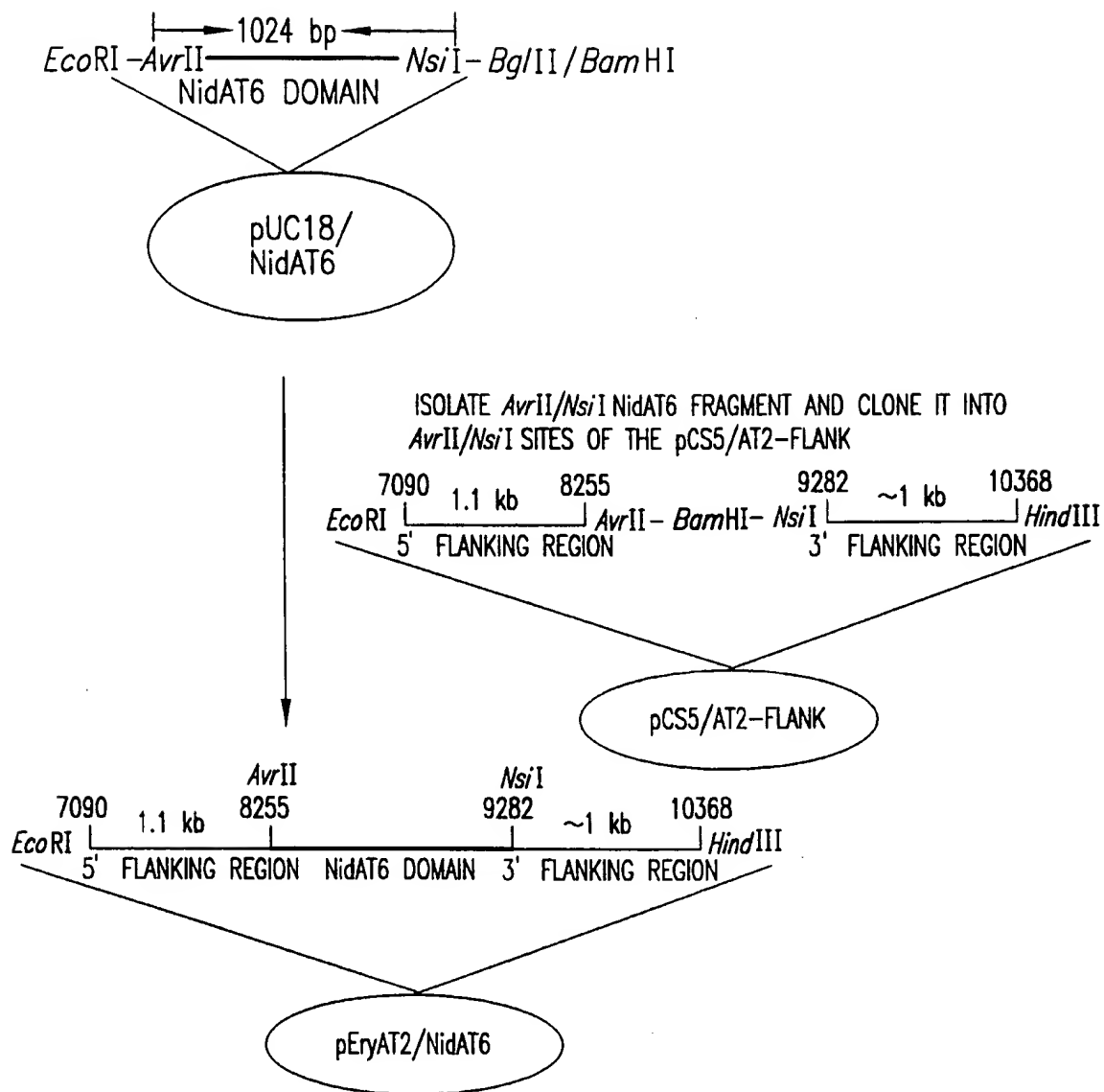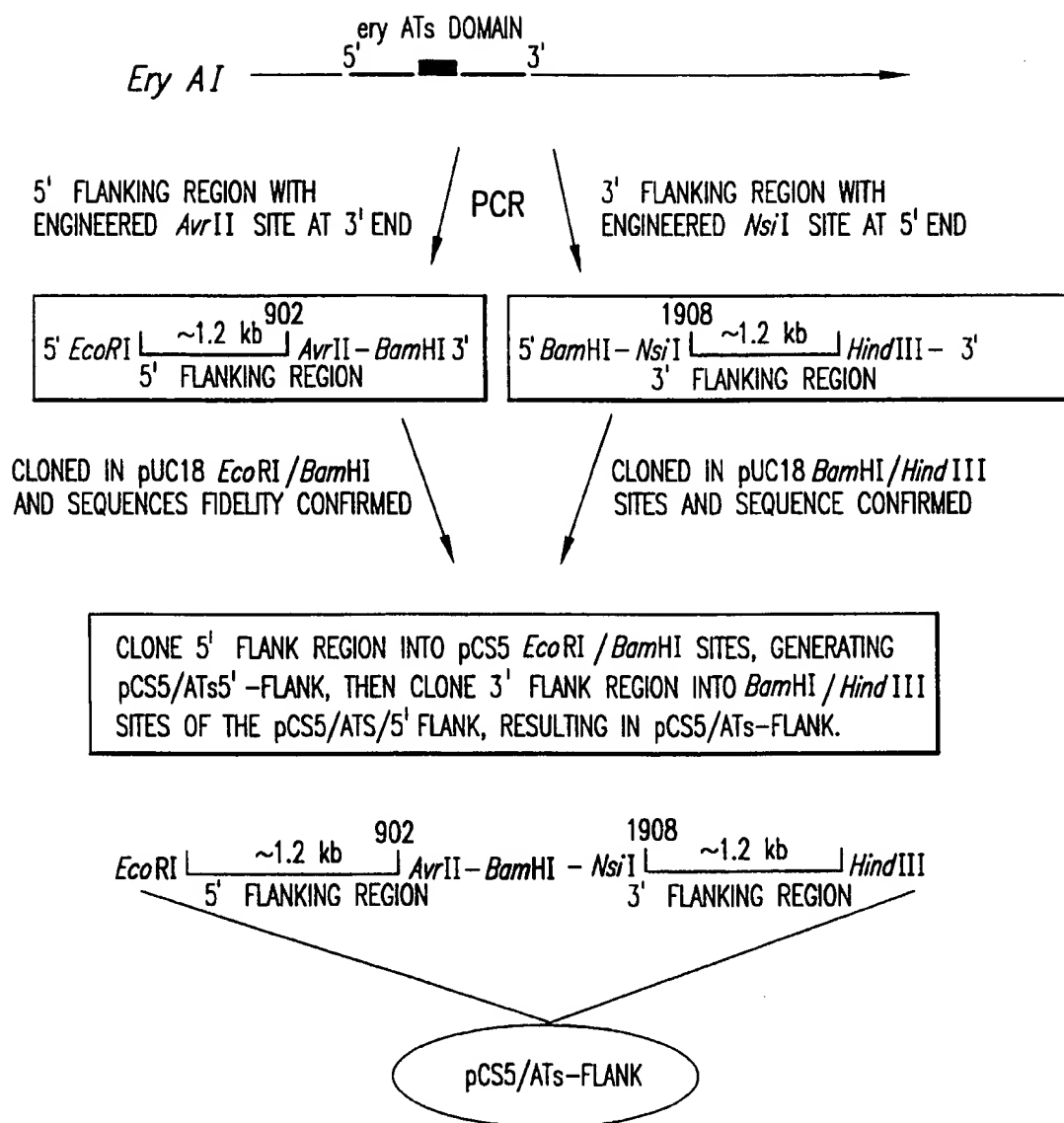NIDDAMYCIN CLUSTER ────────■───────────────────▶

PCR NidAT6 DOMAIN WITH ENGINEERED OLIGOS

├──▶ 1024 bp ◀──┤
5' -*Eco*RI -*Avr*II────────────── *Nsi*I -*Bgl*II-3'
NidAT6 DOMAIN

CLONED INTO pUC18 *Eco*RI /*Bam*HI SITES
AND SEQUENCES FIDELITY CONFIRMED

├──▶ 1024 bp ◀──┤
*Eco*RI -*Avr*II────────────── *Nsi*I - *Bgl* II/ *Bam*HI
NidAT6 DOMAIN

(CLONED NidAT6 DOMAIN WITH
INTRODUCED *Avr*II /*Nsi*I SITES)

pUC18/
NidAT6

FIG.26

FIG.27

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FIG.28

FIG.29

ISOLATE *Avr*II/*Nsi*I NidAT6 FRAGMENT AND CLONE
IT INTO *Avr*II/*Nsi*I SITES OF THE pCS5/AT1–FLANK

FIG.30

FIG.31

FIG.32